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PRINCIPAL INVESTIGATOR: Igor P. Dmitriev, Ph.D.

CONTRACTING ORGANIZATION: The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

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Igor P. Dmitriev, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

E-Mail: idmitriev@gtp.ccc.uab.edu

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The use of adenovirus (Ad) vectors for cancer gene therapy applications is currently limited due to the broad viral tropism associated with the widespread expression of primary coxsackievirus and adenovirus receptor (CAR) in human tissues. To confer Ad targeting capability to relevant cell types we have proposed the use of soluble CAR ectodomain (sCAR) fused with ligand to block CAR-mediated tropism and simultaneously achieve infection through a novel receptor overexpressed on target cells. To target Ad vectors to breast cancer cell types we produced bispecific proteins containing sCAR and either α_v-integrin-binding RGD peptide or single-chain antibody against c-*erb*B-2 oncoprotein. We demonstrated that retargeting of Ad infection to cancer cell types via cellular integrins and c-*erb*B-2 is able to provide tumor-specific enhancement of gene delivery to breast tumor xenografts via the systemic vascular administration route. Our animal experiments established that Ad infection mediated by sCAR-ligand adapter proteins was dependant upon their specificity for breast tumor xenografts and thus supported our hypothesis that tumor-targeted gene delivery could be achieved *in vivo* by the modification of Ad tropism. To our knowledge this is the first demonstration that an Ad vector can be selectively retargeted to tumor xenografts when administered via a peripheral vein.

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INTRODUCTION

Breast cancer is the most common cancer in women worldwide and continues to be a major health problem. It accounts for one-third of cancer diagnoses and 15% of cancer deaths in U.S. This argues strongly for the development of effective strategies to accomplish treatment of breast cancer. Cancer gene therapy has been evaluated as a candidate therapy for a variety of carcinomas to eradicate loco-regional and disseminated disease, which is not adequately addressed by conventional treatments. Viral agents could represent a powerful anticancer treatment platform if they can be designed to infect tumor cells with a requisite level of efficiency and specificity. In this regard, replication-competent adenovirus (Ad) vectors have been of high interest, owing to their ability to propagate in epithelial cells, the origin of most human cancers. However, realization of the full potential of Ad vectors for targeted cancer treatment is currently limited by broad viral tropism which results in widespread tissue distribution of systemically administered Ad with preferential accumulation in the liver (1).

Several studies, including our own work, have established the feasibility of tropism modification of Ad vectors for cell-specific gene delivery (2-6). The distinct aim of the current study was to evaluate the potential for such targeting approaches to maintain specificity in the stringent context of systemic vascular administration. Such a demonstration would be a key milestone for the development of targeted Ad vectors for gene therapy and have important implications for treatment of carcinoma of the breast.

BODY

Task 1. To design bispecific soluble sCAR-ligand fusion proteins possessing the capacity to achieve high efficacy binding both to Ad fiber protein and surface receptor on breast cancer cells.

Rationale. It was shown that soluble extracellular domain of CAR bound to representatives of all Ad subgroups except subgroup B (7). Both structural analysis of fiber knob-CAR complexes and knob domain mutagenesis showed that there are three CAR-binding sites on the fiber knob domain (8, 9). Further kinetic analysis of Ad2 fiber knob binding to the CAR D1 domain revealed that an avidity mechanism corresponds to trimeric receptor-ligand interaction (10). To take an advantage of trivalent nature of CAR-knob binding for Ad targeting, we proposed to derive recombinant protein consisting of soluble CAR in fusion with trimerization sequence and a targeting ligand. Our goal was to generate a trimeric sCAR-ligand molecules that could provide high affinity linkage between Ad vector and cellular target receptor. Predicted CAR-knob binding ratio may increase linkage efficiency of trimeric sCAR-ligand fusion proteins with virus particles and thereby contribute to the ligand-mediated binding of such viral complexes to target receptors. In order to achieve trimerization of sCAR-fusion molecules, we choose to employ polypeptide derived from bacteriophage T4 fibritin protein forming highly stable homotrimers. As targeting ligands we proposed to use an α_v-integrin-binding RGD-4C peptide (CDCRGDCFC) and NGR peptide motif (CNGRCVSGCAGRC) initially isolated by phage biopanning (11). Phage displaying an RGD-containing peptide when injected intravenously into tumor-bearing mice was detected in melanoma and breast carcinoma tumor blood vessels, but not in normal tissues (12). NGR peptide coupled with doxorubicin was shown to have more potent effects against metastatic human breast cancer xenografts than the free drug (13). TNF fused with CNGRC peptide also was shown to induce stronger antitumor effects in animal models than TNF alone (14). To produce CAR-ligand fusion proteins a baculovirus expression system that has already proved its utility for the expression of functional soluble CAR (7) and sCAR-EGF chimera (6) was used.

a. Engineer an universal derivative of plasmid shuttle vector containing under the control of polyhedrin promoter DNA sequences coding for: soluble CAR protein-six, His tag, flexible linker and a multiple cloning site (MCS) for cloning DNA sequences of targeting ligands.

The donor plasmid for the generation of recombinant baculoviruses expressing sCAR-ligand fusion proteins was made as follows. To generate a recombinant gene encoding the extracellular domain of human CAR followed by polypeptide sequence derived from bacteriophage T4 fibritin protein (15), PCR was used. Sense primer 5' GTT GAA AGA TCT GGA TTA ACC AAT AAA ATA AAA GCT ATC GAA ACT GAT ATT GCA TCA G complementary to the position 1240 of the fibritin gene was designed to introduce *Bgl*II restriction site into amplified DNA sequence, and antisense primer 5' TTG CGG CCC CAG CGG CCG CTG GTG ATA AAA AGG TAG complementary to the position 16 of untranslated 3'-region was designed both to introduce *Not*I and substitute stop codon for alanine (GCC) codon. The PCR-derived 238-bp DNA fragment was digested with *Bgl*II and *Not*I, and 214-bp DNA fragment encoding 71 carboxy-terminal amino acids (aa) of fibritin M polypeptide (15) was purified. To construct the recombinant gene coding for extracellular CAR, six histidines, and short flexible linker fused with fibritin coding sequence *Bgl*II-*Not*I-fragment was ligated with plasmid pFBshCAR-EGF (6) digested with *Bam*HI and *Not*I substituting EGF for fibritin. Resultant plasmid designated pFBsCARf was cleaved with *Not*I and ligated with oligonucleotide duplex 5' GGC CCA ACC GCA GCC AAA ACC TCA ACC CCA GCC ACA ACC TCA GCC CAA ACC TCA GCC TAA ACC GGT TTA AAC GGC C coding for proline-rich hinge region derived from camel immunoglobulins and containing *Age*I site followed by stop codon. Plasmid clone containing hinge DNA fragment in correct orientation was selected by sequencing and designated pFBsCARfCh. The constructed plasmid was then used as a vector to generate the recombinant baculovirus to produce sCARf control protein. Oligonucleotides 5' CCG GGA GCT CTG CGC TAG CT and 5' CCG GAG CTA GCG CAG AGC TC designed to contain *Nhe*I site and AgeI compatible cohesive 5'-ends were annealed to form DNA duplex and ligated with *Age*I-digested pFBsCARfCh. Plasmid clones were sequenced, and the plasmid containing the duplex in the correct orientation was designated pFBsCARfMCS. The resultant plasmid contains recombinant gene coding for sCAR, six-His tag, flexible linker, fibritin polypeptide, proline-rich hinge, and multiple cloning site (MCS) to facilitate downstream incorporation of ligand sequences (Fig. 1).

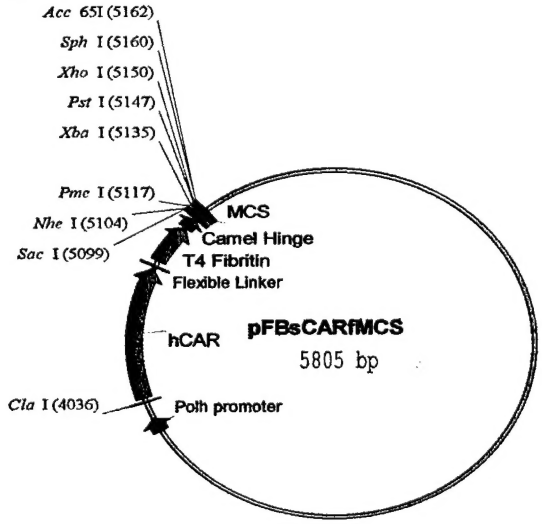

Fig. 1. Circular map of the plasmid vector pFBsCARfMCS. Plasmid contains recombinant gene encoding human CAR ectodomain (hCAR), flexible linker, trimerization domain from phage T4 fibritin protein (T4 fibritin), proline-rich hinge region from camel immunoglobulins, and multiple cloning site for

incorporation of ligand sequences (MCS) under control of baculovirus polyhedrin promoter (Polh promoter).

b.- c. Design oligonucleotides coding for short peptide ligands RGD-4C (CDCRGDCFC) and NGR (CNGRCVSGCAGRC). Construct a plasmid vectors coding for the sCAR-RGD-4C and sCAR-NGR fusion proteins and create recombinant baculoviruses that express corresponding proteins in infected insect cells.

The plasmids coding for the sCAR fused with either RGD-4C or NGR peptide motifs were made as follows. To incorporate the RGD-4C peptide into the carboxy terminus of sCARf fusion protein, oligonucleotides 5' TGC GAC TGT CGC GGG GAT TGC TTT TGT GG 3' and 5' CTA GCC ACA AAA GCA ATC CCC GCG ACA GTC GCA AGC T 3' were designed to form DNA duplex coding for A*CDCRGDCFC*G followed by in-frame stop codon. To incorporate the NGR peptide, oligonucleotides 5' TGC AAC GGA AGG TGT GTAAGC GGG TGC GCG GGC AGA TGC GG 3' and 5' CTA GCC GCA TCT GCC CGC GCA CCC GCT TAC ACA CCT TCC GTT GCA AGC T 3' were designed to form DNA duplex coding for A*CNGRCVSGCAGRC*G and stop codon. In addition, both generated DNA duplexes contained *Sac*I- and *Nhe*I-compatible cohesive ends designed to fuse the sCAR-fibritin ORF with peptide coding sequences. The oligonucleotide duplexes were cloned into *Sac*I and *Nhe*I-digested pFBsCARfMCS, DNA clones were sequenced in the region of insert and the plasmids containing RGD-4C and NGR coding sequences in the correct orientation were designated pFBsCARf-RGD4C and pFBsCARf-NGR respectively. The constructed plasmids encoding sCAR-fibritin protein fused with peptide ligands were then used as vectors to generate the recombinant baculovirus genomes using the Bac-to-Bac baculovirus system (Life Technologies, Grand Island, N.Y.). To produce sCARf-RGD4C and sCARf-NGR fusion proteins, generated recombinant baculoviruses were used to infect insect cells.

d. Use recombinant baculoviruses to infect Hi5 insect cells in order to express and purify sCAR-ligand proteins in a preparative amounts.

i. Expression and purification of sCAR-fusion proteins. The fusion proteins sCARf, sCARf-RGD4C and sCARf-NGR were expressed in High Five cells (Invitrogen, Carlsbad, Calif.) infected with recombinant baculoviruses as described previously (6). Baculovirus infections resulted in high expression levels of secreted soluble sCARf, sCARf-RGD4C and sCARf-NGR fusion proteins. Secreted His_6-tagged proteins were purified from dialyzed culture medium by immobilized metal ion affinity chromatography (IMAC) on Ni-nitrilotriacetic acid (NTA)-Sepharose (Qiagen, Valencia, Calif.) as recommended by the manufacturer. Protein concentrations were determined by the BCA-200 protein assay kit (Pierce, Rockford, IL) with bovine gamma globulin as the standard. Purified sCARf, sCARf-RGD4C and sCARf-NGR proteins were then analyzed for trimerization and presence of encoded polypeptide sequences.

ii. Structure of recombinant sCAR-fusion proteins. To characterize structure and composition of recombinant proteins gel electrophoresis and Western blot were used. To determine whether generated sCAR-fusion proteins could form trimers, these proteins were analyzed by SDS-PAGE. Electrophoretic separation of boiled protein samples showed the presence of major bands migrating as was expected for monomeric forms of sCARf, sCARf-RGD4C, and sCARf-NGR proteins with molecular masses of 36.0, 36.7 and 37.0 kDa respectively (Fig. 2). Electrophoretic mobility of unboiled protein samples was decreased and close to those predicted for trimeric forms of sCARf, sCARf-RGD4C, and sCARf-NGR proteins. This result demonstrated that fibritin polypeptide incorporated in the context of recombinant molecule is capable of providing an efficient trimerization of sCAR-fusion proteins.

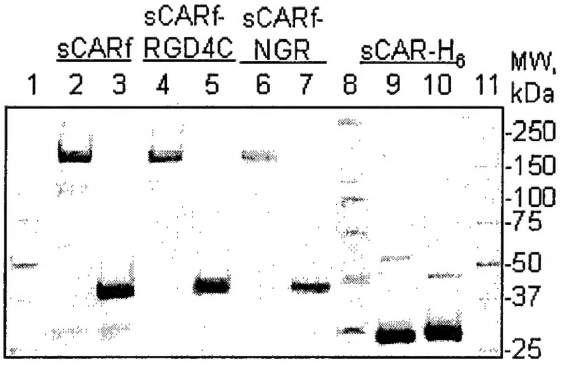

Fig. 2. Trimerization analysis of sCAR-fusion proteins by polyacrylamide gel electrophoresis. Samples of purified sCARf (lanes 2, 3), sCARf-RGD4C (lanes 4, 5), sCARf-NGR (lanes 6, 7), sCAR-H_6 (lanes 9, 10), and molecular mass markers (lanes 1, 8, 11) were separated on 4-15% gradient SDS gel. The samples in lanes 3, 5, 7, and 10 were boiled in Laemmli loading buffer to denature proteins to monomers, while lanes 2, 4, 6, and 9 contain unboiled proteins in their native conformation. Protein bands were visualized by GELCODE blue stain reagent (Pierce, Rockford, IL). The numbers on the right indicate molecular masses of marker proteins in kilodaltons (kDa).

Western blot analysis of protein composition using monoclonal antibodies to sCAR protein revealed the presence of CAR ectodomain in the context of each sCAR-fusion protein (Fig. 3).

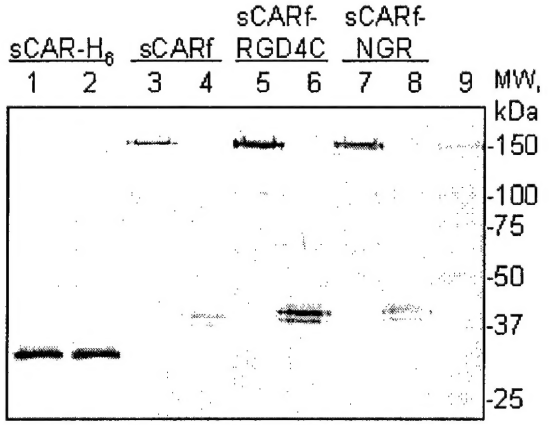

Fig. 3. Western blot analysis of sCAR-fusion proteins. Samples of purified sCAR-H_6 (lanes 1, 2), sCARf (lanes 3, 4), sCARf-RGD4C (lanes 5, 6), sCARf-NGR (lanes 7, 8) were boiled in Laemmli loading buffer and separated on 4-15% gradient SDS gel. Electrophoretically resolved proteins were transferred to PVDF membrane probed with MAb to soluble CAR ectodomain and then detected with goat anti-mouse antibody-Alkaline Phosphatase conjugate (Sigma). The numbers on the right indicate molecular masses of marker proteins (laine 9) in kilodaltons (kDa).

These results suggest that derived proteins are able to maintain both designed composition and stable trimeric structure. Thus, we obtained preparative amounts of purified sCARf, sCARf-RGD4C and sCARf-NGR trimeric fusion protein for subsequent experiments.

iii. Analysis of sCAR-fusion proteins binding to Ad5 fiber knob. We first chose to characterize trimeric sCARf protein with respect to its ability to bind Ad fiber knob domain compare to sCAR-His_6 monomer generated previously (6). To compare fiber knob binding efficiencies of sCARf and sCAR-His_6 we used an ELISA. This assay showed that trimeric sCARf protein efficiently bound to immobilized Ad5 fiber knob (Fig. 4).

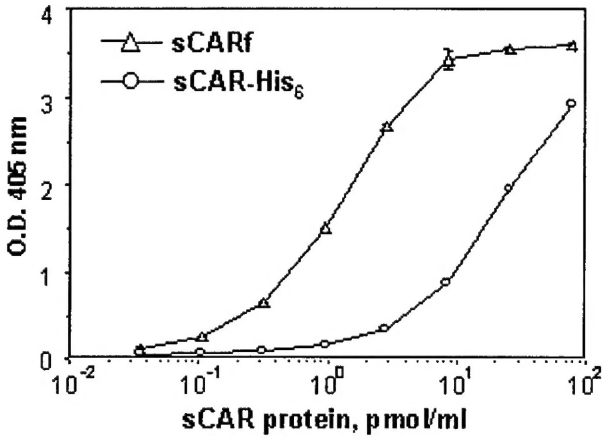

Fig. 4. ELISA analysis of sCAR-fusion proteins binding to Ad5 fiber knob. Purified trimeric sCARf and monomeric sCAR-His_6 proteins were incubated at various concentrations with recombinant Ad5 fiber protein immobilized on an ELISA plate. Bound sCAR-fusion proteins were probed with MAb against soluble CAR ectodomain and then detected with goat anti-mouse antibody-Alkaline Phosphatase conjugate (Sigma). Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting standard deviations are smaller than the symbols.

Compare to the monomeric sCAR-His_6 control protein, the affinity of sCARf-knob binding was greatly increased in a wide range of used concentrations. Based on the obtained result, whereby generated sCARf protein is able of high affinity binding to fiber knob domain, we hypothesized that trimeric sCAR-fusion proteins may be of utility to block CAR-mediated virus-cell attachment and Ad infection.

iv. Analysis of sCAR-fusion proteins ability to block Ad infection. In order to evaluate whether improved knob binding results in increased ability of trimeric sCAR-fusion proteins to block Ad infection, we performed an infection inhibition assay. Recombinant AdCMVLuc vector expressing luciferase gene was preincubated with one of the sCAR-His_6, sCARf-RGD4C, or sCARf-NGR at increasing concentrations or PBS and used to infect 293 cells known to express high level of CAR (Fig. 6). The ability of sCAR fusion proteins to block viral infection was assessed by sCAR protein dose dependent impairment of Ad-mediated gene transfer as measured by luciferase activity of infected cells (Fig. 5). It was shown that, compare to monomeric sCAR-His_6 protein, both sCARf-RGD4C and sCARf-NGR displayed an increased ability to inhibit CAR-mediated Ad infection. The concentrations of sCARf-RGD-4C, sCARf-NGR and sCAR-His_6 needed to compete Ad infection by 50% were 3, 6, and 54 nM respectively. This experiment demonstrated that trimeric sCAR-fusion proteins possessed superior ability to inhibit Ad infection in comparison with sCAR-His_6 monomer. This finding validated the utility of produced sCAR-RGD4C and sCARf-NGR trimeric fusion proteins in order to block Ad native tropism and, therefore, provided a rationale for further Ad targeting studies.

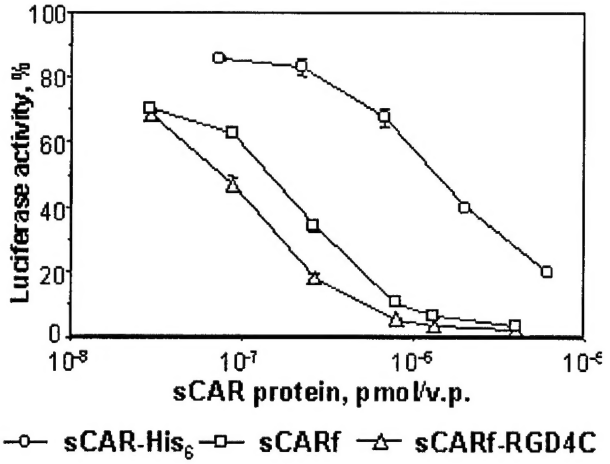

Fig. 5. Inhibition of Ad-mediated gene transfer. AdCMVluc vector was mixed with either PBS or varying amounts of sCAR-His_6, sCARf or sCAR-RGD4C fusion proteins and incubated for 15 min at room temperature. Viral mixtures were diluted to 1 ml with infection medium and 200-µl aliquots were added to monolayers of 293 cells grown in a 24-well plate (5×10^5 cells/well) at MOI of 100 v.p./cell. After 45-min incubation at room temperature to allow virus internalization infection medium was aspirated, the cells were washed with PBS, and a growth medium was added. The cells were incubated at 37°C to allow expression of the reporter genes. Twenty four hours postinfection the cells were lysed and luciferase activity was analyzed by using the Promega (Madison, Wis.) luciferase assay system and a Berthold (Gaithersburg, Md.) luminometer. Luciferase activities detected in cells infected in the presence of sCAR-fusion proteins are shown as percentages of luciferase activity registered in control cells infected with AdCMVLuc mixed with PBS. Each point represents the cumulative mean ± SD of triplicate determinations. Some error bars depicting standard deviations are smaller than the symbols.

Task 2. Evaluate the ability and efficacy of sCAR-ligand fusion proteins for targeted gene delivery to breast carcinoma cells *in vitro.*

a. Evaluation of ability of sCAR-ligand fusion proteins to bind to the receptors overexpressed on breast cancer cells using binding assays.

The expression levels of CAR and α_V-integrins, a putative receptor for the RGD-4C motif, were determined for a number of established breast adenocarcinomas and described in previous report. Here we demonstrated expression of APN, which was shown to serve as receptor for the NGR motif (16)for a set of cell lines established from breast cancer and endothelial HUVEC cells. The screening of ten breast cancer cell lines revealed a significant level of APN only on MDA-MB-468 cells. Fig. 6 shows representative results of indirect immunofluorescence assay for APN expression in MDA-MB-468, MCF-7, MDA-MB-435S, and HUVEC cells.

The use of sCARfRGD and sCARfNGR adapter proteins may augment infection efficiency by facilitating virus binding to cellular integrins and APN, respectively, in breast cancer cells normally refractory to Ad due to lack of CAR To test this hypothesis we studied the ability of sCARfRGD and sCARfNGR to mediate binding of ^{3}H-radiolabeled Ad to MB-468, MCF-7, MB-435S breast cancer cells and endothelial HUVEC cells. To determine the improvements in virus cell-binding ^{3}H-Ad was complexed with dilutions of sCARfRGD, sCARfNGR, or sCARf protein as a control and allowed to bind cells at 4°C to prevent virus internalization. Detected cell-bound radioactivities were dependent on sCAR-ligand protein dose and were significantly higher for ^{3}H-Ad complexed with sCARfRGD compered to ^{3}H-Ad alone.

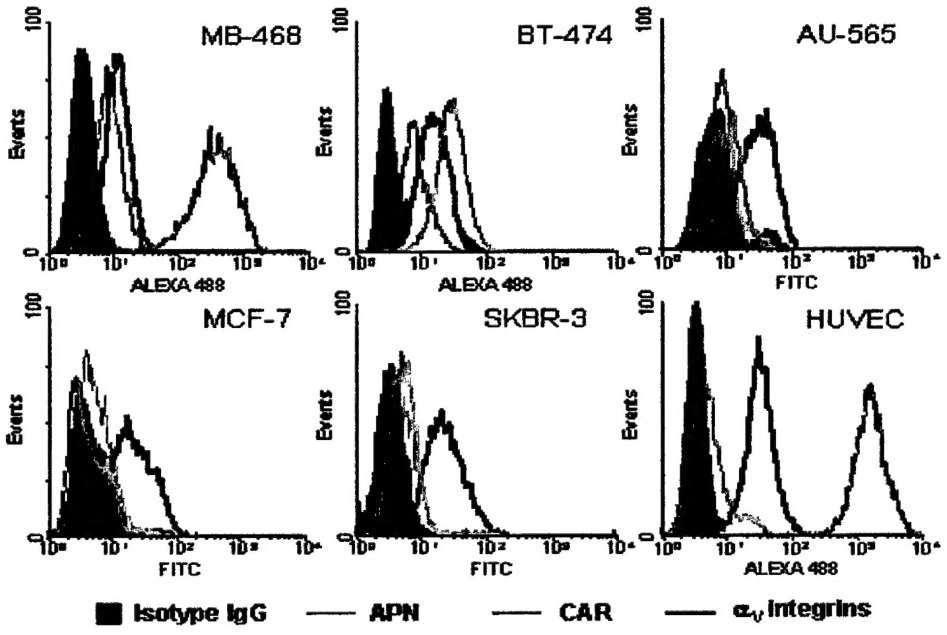

Fig. 6. Expression of APN, CAR, and α_V-integrins in breast cancer cell lines. The cell lines MDA-MB-468, BT-474, AU-565, MCF-7, SK-BR-3, and endothelial cell line HUVEC were analyzed for expression of APN, CAR, and α_V-integrins by indirect immunofluorescence assay using anti-CD13 WM15, anti-CAR RmcB, and anti-α_V-integrins MAB1953 monoclonal antibodies, respectively. Positive staining for APN (*blue line*), CAR (*gray line*), and α_V-integrins (*black line*) is seen relative to an isotype control IgG (*spike filled in black*). Representative data from two independent experiments are shown.

As shown in Fig. 7, Ad complexed with sCARfRGD resulted in 2-, 9-, 60-, and 2-fold increase of ^{3}H-Ad binding to MB-468, MCF-7, MB-435S, and HUVEC, respectively.

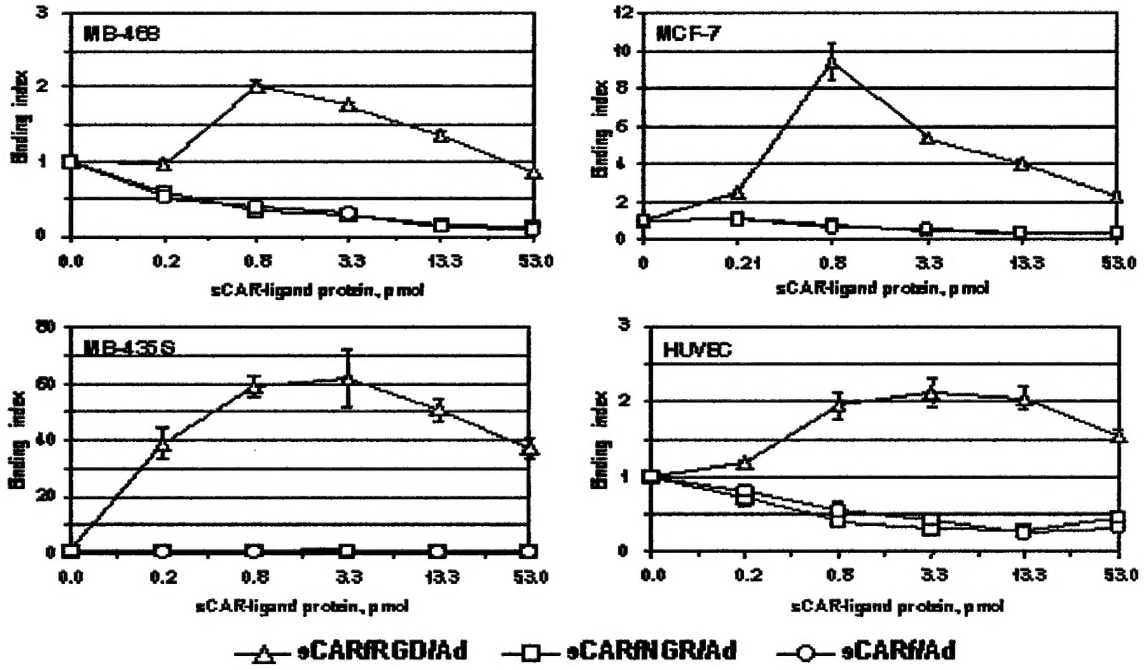

Fig. 7. Comparison of ^{3}H-labeled Ad binding to MDA-MB-468, MCF-7, MDA-MB-435S, and HUVEC cells. ^{3}H-labeled Ad was preincubated for 30 min at room temperature with different

amounts of sCARfRGD, sCARfNGR or sCARf as control. ^{3}H-Ad/sCAR-ligand mixtures (10^{5} cpm per sample) were then added to cells aliquots (10^{6}) and allowed to bind for 1 h at 4°C. Bound radioactivity was determined after pelleting the cells by centrifugation. Binding indices were calculated from the ratio of the mean bound radioactivity of ^{3}H-Ad preincubated in presence of sCAR-ligand versus ^{3}H-Ad preincubated in absence of sCAR-ligand protein. Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

In contrast, virus complexed with either sCARfNGR or sCARf control protein inhibited virus cell binding compared to Ad alone. These data clearly demonstrate that, in contrast to sCARfNGR, the sCARfRGD adapter protein is capable of enhancing virus binding to cellular integrins and confirm that formation of the Ad/sCARfRGD complexes provide improved of Ad binding to breast cancer cells.

To further refine and extend this approach for particular breast cancer cell types we have engineered the sCARfC6.5 adapter containing the C6.5 scFv with binding specificity for c-*erb*B-2 oncoprotein (5). To validate sCARfC6.5 protein binding to cellular c-*erb*B-2 we employed an indirect immunofluorescence assay. The sCARfC6.5 protein was allowed to bind AU-565 breast cancer cells overexpressing c-*erb*B-2 and then was detected by incubation with a primary anti-CAR RmcB antibody followed by a secondary antimouse fluorochrome-conjugated antibody. The MDA-MB-468 breast

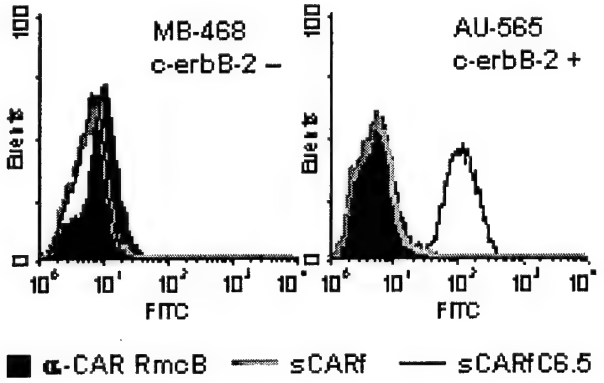

Fig. 8. Confirmation of sCARfC6.5 protein binding to cellular c-*erb*B-2. Trimeric sCARfC6.5 and sCARf fusion proteins were incubated with either c-*erb*B-2-positive AU-565 or c-*erb*B-2-nagative MB-468 cells. The sCAR fusion proteins bound to cells were probed with anti-CAR RmcB MAb and then detected with secondary Alexa 488-labeled goat antimouse antibodies. Binding of sCARfC6.5 protein *(black line)* to c-*erb*B-2-positive AU-565 cells is seen because of the positive staining relative to sCARf control protein *(gray line)* or anti-CAR MAb alone *(spike filled in black)*. Representative data from two independent experiments are shown.

cancer cells, shown to be c-*erb*B-2-negative, were used as a control. As shown in Fig. 8, incubation of AU-565 cells, naturally low in CAR (Fig. 6), with sCARfC6.5 protein increased cell binding of anti-CAR MAb. In contrast, neither the incubation of AU-565 cells with sCARf control protein nor the incubation of sCARfC6.5 with MDA-MB-468 c-*erb*B-2-negative cells revealed any increase of anti-CAR antibody binding compared with control. Thus, we demonstrated the C6.5 scFv that was incorporated in the context of the fusion protein retained its functional ability to recognize cellular c-*erb*B-2 oncoprotein, which enabled sCARfC6.5 protein binding to c-*erb*B-2-positive cells.

b. Confirmation of specificity of ligand-receptor binding by blocking with free ligand or specific anti-EGFR monoclonal antibodies in competition-binding assay.

We previously showed that Ad complexed with an sCAR-EGF adapter protein overcomes the barrier of inefficient gene transfer to specific cancer cell types (6). As illustrated in Fig. 9, augmentation of radiolabeled Ad binding to EGFR-positive A-431 cells mediated by sCAR-EGF adapter could be competitively blocked by preincubation of the cells with either human EGF or anti-EGFR MAb, confirming specificity of sCAR-EGF binding to EGFR. In this study we have demonstrated that highest level of Ad binding to MB-468 and MCF-7 cells was achieved at 0.8 pmol of sCARfRGD protein per $1.5x10^8$ vp while 3.3 pmol was required to reach maximum for MB-435S and HUVEC cells (Fig. 7). It is noteworthy that increases in the sCARfRGD/virus ratio proved inhibitory to binding, likely due to excess of free sCARfRGD protein and its competition for integrin binding.

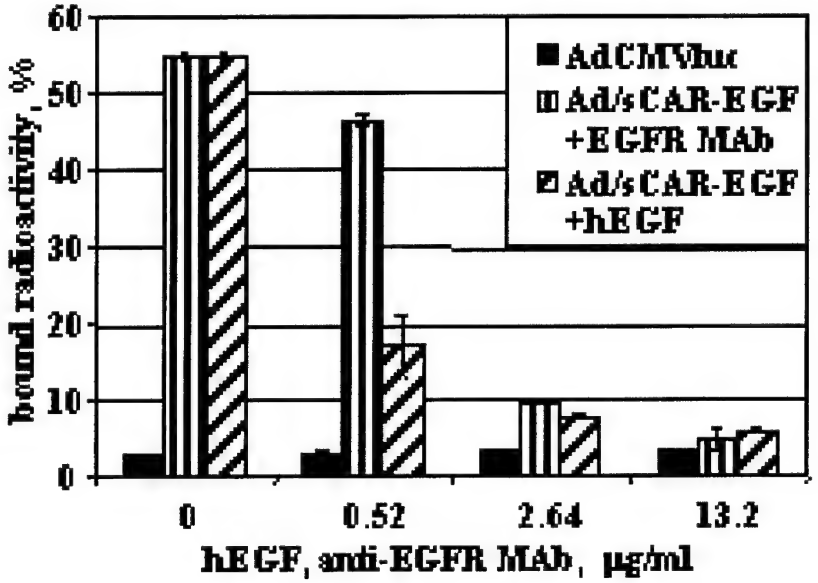

Fig. 9. Specific inhibition of sCAR-EGF-mediated Ad binding. ^{3}H-labeled Ad was preincubated for 30 min at room temperature with 0.4 mg of sCAR-EGF. Human epidermoid carcinoma A-431 cells, overexpressing EGFR, were preincubated for 30 min at 4°C in the presence or absence of either human EGF or anti-EGFR antibody at different concentrations (0.52 to 13.2 mg/ml). ^{3}H-Ad/sCAR-EGF samples (10^5 cpm) were then added and allowed to bind for 1 h at 4°C. Cells were washed by centrifugation, and radioactivities of cell pellets were determined in a beta counter. Data are presented as the percentage of input ^{3}H-Ad bound after washing and calculated as the cumulative mean ±SD of triplicate determinations.

c. Demonstration of capacity of sCAR-ligand fusions to target Ad to receptors on breast cancer cells using gene transfer assay. Determination of optimal sCAR-ligand/Ad molar ratio that gives maximal gene transfer.

Having established that the sCARfRGD adapter demonstrates significant ability to increase Ad binding to CAR-deficient breast cancer cells, we investigated its ability to improve viral infection via CAR-independent pathway. To test the utility of sCAR-ligand proteins for Ad targeting, we used both sCARfRGD and sCARfNGR proteins in Ad-mediated gene transfer assay with established human breast cancer cell lines and endothelial HUVEC cells. Our study showed that the majority of breast cancer cells are relatively refractory to Ad infection (Fig. 10). These data were corroborated by flow cytometry analysis that showed either absence or low level of CAR on their cell surface. Importantly, high levels of integrins and c-*erb*B-2 (5) were

detected in these cell lines, which suggested that Ad targeting to these receptors might overcome poor vector susceptibility attributable to the lack of CAR.

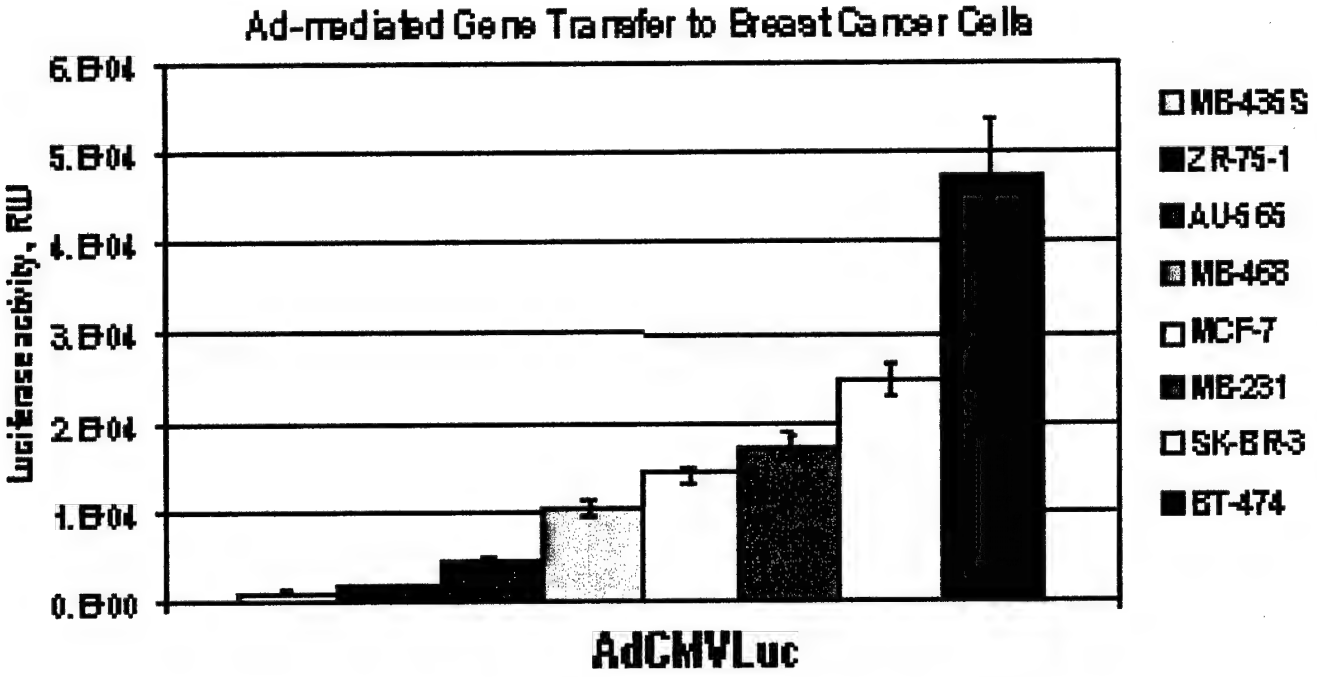

Fig. 10. Ad-mediated gene transfer to breast cancer cells. The breast cancer cell lines MDA-MB-435S, ZR-75-1, AU-565, MDA-MB-468, MCF-7, MB-231, SK-BR-3, and BT-474 were infected with AdLucGFP vector expressing luciferase reporter gene. Cells were incubated for 40 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as relative light units (*RLU*) detected in the cells. Each point represents the cumulative mean ±SD of triplicate determinations.

To determine the magnitude of gene transfer augmentation provided by sCAR-ligand proteins they were titered against a constant dose of AdLucGFP vector (MOI 100 vp/cell) as measured by improvements in reporter gene transfer efficiency. The increase of Ad infection efficiency mediated by sCARfRGD or sCARfNGR adapters (targeted Ad) was measured by luciferase activity that was detected in infected cells compared with untargeted Ad preincubated with sCARf control protein. As shown in Fig. 11, the sCARfRGD targeting protein mediated a 3-, 4-, 6-, 8-, 11-, 13-, and 120-fold enhancement of gene transfer to BT-474, MB-468, SK-BR-3, HUVEC, AU-565, MCF-7, and MB-435 cells, respectively. Consistent with the results of binding experiments, we did not observe any significant augmentation of viral gene transfer in case of sCARfNGR. As expected, the enhanced Ad binding to cells mediated by the sCARfRGD targeting adapter correlated with augmentation of infection efficiency, as seen in gene transfer experiments. The sCARfRGD/Ad ratio providing maximal gene transfer increase ranged from $1x10^{-7}$ to $3x10^{-7}$ pmol/vp for most cell lines tested.

The magnitude of gene transfer augmentation by c-*erb*B-2-targeted Ad complexed with sCARfC6.5 adapter was illustrated on selected c-*erb*B-2-positive cell lines and c-*erb*B-2-negative MDA-MB-468 cells (5). It was shown that sCARfC6.5 targeting protein mediated a 3.4-, 11-, 32-, 47-, and 135-fold enhancement of gene transfer to MCF-7, SK-OV-3, BT-474, SK-BR-3, and AU-565 cells, respectively. Consistent with the augmentation of the Ad gene transfer to c-*erb*B-2-positive cell lines achieved by sCARfC6.5 targeting protein, sCARf control protein caused a marked decrease in Ad gene transfer. Importantly, the use of both targeting and control protein for Ad infection of c-*erb*B-2-negative MDA-MB-468 cells that express moderate levels of CAR resulted in an 8-fold decrease of gene transfer. These

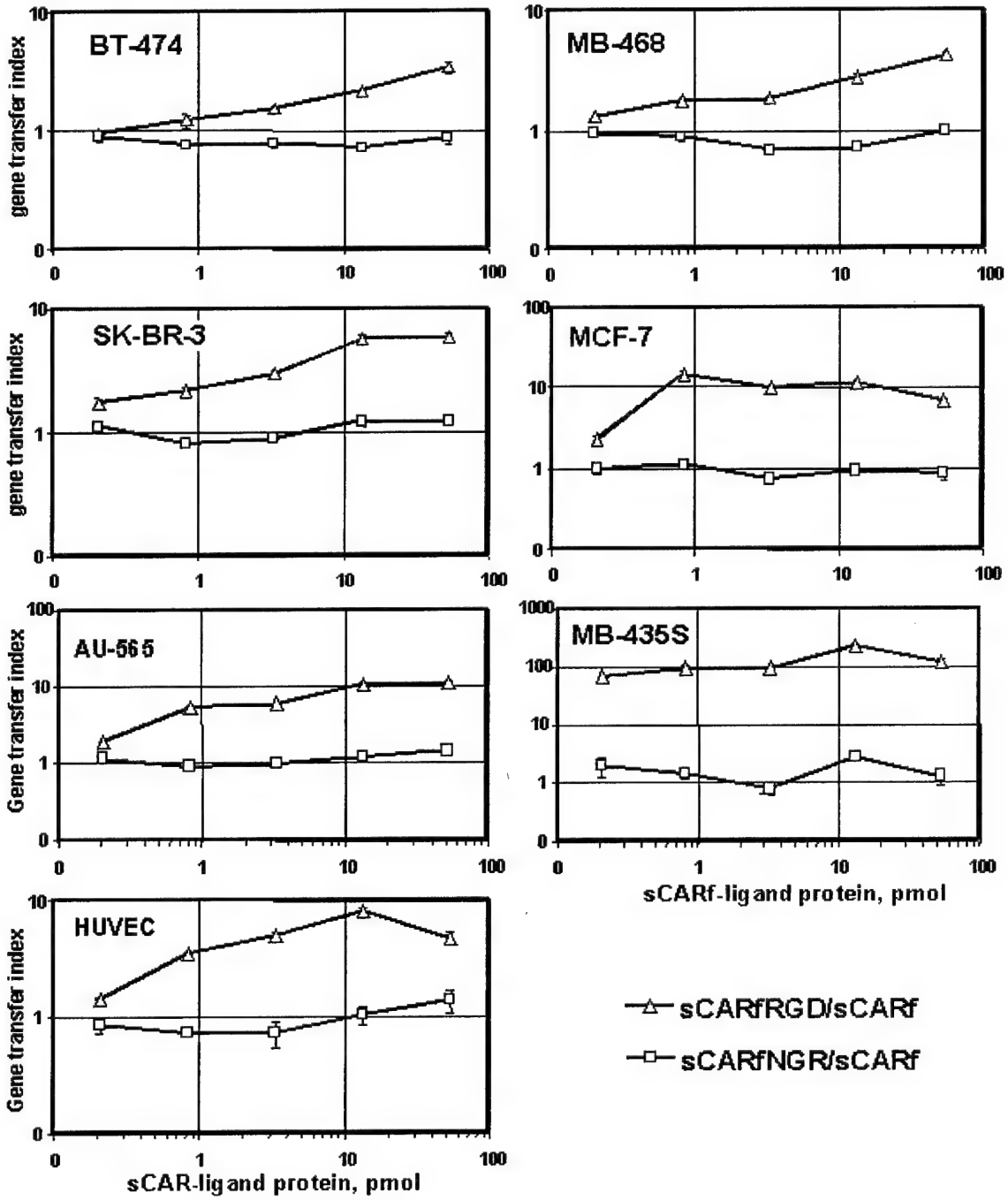

Fig. 11. Determination of optimal sCAR-ligand protein:Ad ratio. AdLucGFP vector expressing luciferase reporter gene was complexed with sCARfRGD or sCARfNGR targeting proteins or sCARf control protein at varying concentrations to form integrin-targeted, APN-targeted, or untargeted viral complexes, respectively. Monolayers of BT-474, MDA-MB-468, SK-BR-3, MCF-7, AU-565, MDA-MB-435S, and HUVEC cells were infected with targeted or untargeted viral complexes at a MOI of 100 vp/cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was measured. Results are presented as gene transfer indexes that were calculated as the ratio of luciferase activities detected in the cells infected with targeted Ad to luciferase activities detected in the cells infected with untargeted Ad complexes formed at the same concentration of each sCAR protein (*Targeted Ad:Untargeted Ad*). Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.
data clearly indicate that the sCARfC6.5-targeting adapter promoted Ad infection of CAR-deficient cells specifically via a c-*erb*B-2-dependent pathway.

d. Validation of specificity of sCAR-ligand-mediated Ad gene transfer by blocking with free ligand or specific anti-receptor antibodies in an infection-inhibition assay.

Our primary goal is to achieve Ad targeting to breast cancer cells through receptors alternative to CAR. In this regard, the level of c-*erb*B-2-targeted viral infection could be determined by blocking Ad/sCARfC6.5 infection with a specific competitor. As was observed previously, exceeding the optimal sCAR-ligand/virus ratio proved to be inhibitory to gene transfer, apparently because of competition for cellular receptors by free sCAR-ligand protein.

To validate that sCARfC6.5-mediated virus-cell interactions occur specifically via c-*erb*B-2 we carried out an infection-inhibition assay using C6.5 scFv against c-*erb*B-2 as a competitor. Varying amounts of sCARfC6.5 were used to form Ad/sCARfC6.5 complexes prior to infection of AU-565 cells in the presence or absence of C6.5 scFv. As shown in Fig. 12, Ad gene transfer in the presence of competitor was increased 2.2-fold compared to Ad alone. In contrast, the augmentations of Ad gene transfer achieved by increasing amounts of sCARfC6.5 protein were blocked to the level of Ad alone by the presence of free C6.5 scFv. This result indicates that increases in Ad gene transfer efficiency mediated by sCARfC6.5 targeting protein occur through specific interaction with the c-*erb*B-2 oncoprotein.

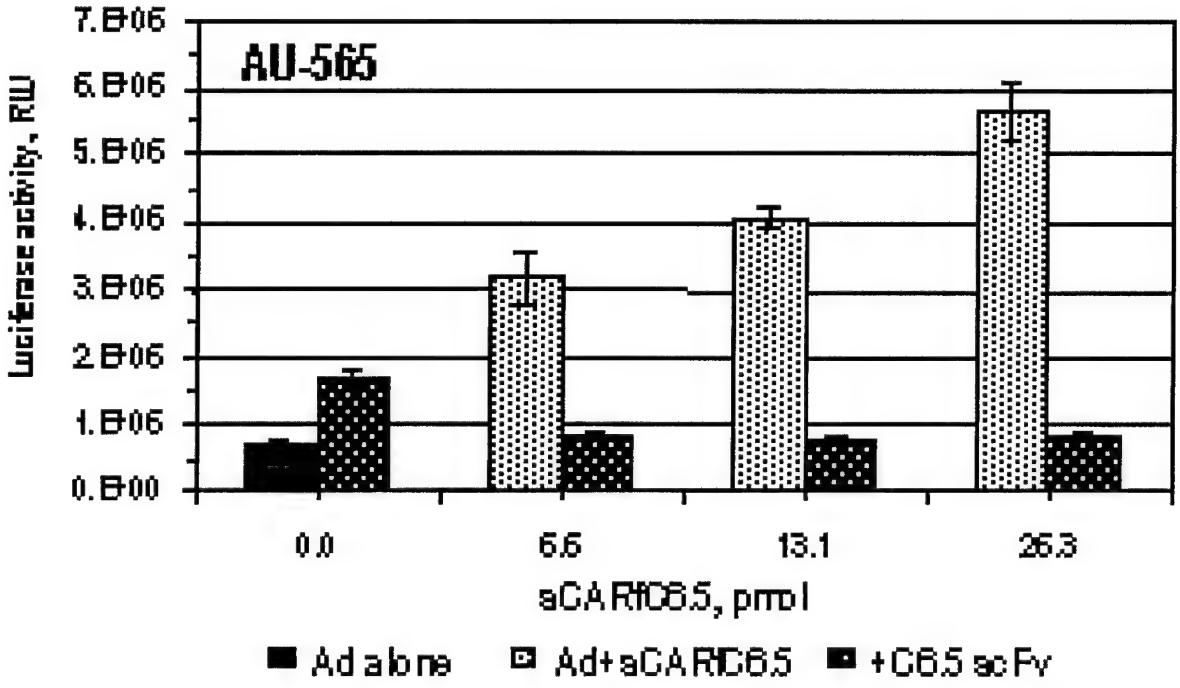

Fig. 12. Specific inhibition of sCARfC6.5-mediated Ad gene transfer. AdLucGFP vector expressing luciferase reporter gene was preincubated with PBS (*Ad alone*) or sCARfC6.5 targeting protein at varying concentrations to form c-*erb*B-2-targeted viral complexes. The monolayers of AU-565 were preincubated for 15 min at 25°C in the presence or absence of C6.5 scFv (5 μg/well) and then were infected with either targeted viral complexes or Ad alone at MOI of 100 vp/cell for 45 min at 25°C. Cells were incubated for 40 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as relative light units (*RLU*) detected in the cells infected in the presence or absence of C6.5 scFv. Each point represents the cumulative mean ±SD of triplicate determinations.

In order to further validate the specificity of Ad targeting using sCAR-ligand adapter proteins we carried out an infection assay in a mixed cell culture. Ad vector expressing red fluorescent protein was preincubated with increasing amounts of sCARfC6.5 or PBS as a

control and then used to infect the cell monolayers formed by Hela and AU-565 breast cancer cells. Importantly, AU-565 cells were shown to have high c-*erb*B-2 expression and absence of CAR while Hela cells express high level of CAR and no c-*erb*B-2. Infected cells were harested 24 hours post-infection and were stained for c-*erb*B-2 using indirect immunofluorescence assay in a parallel with cell mixtures infected with Ad alone and uninfected cells. Stained cell samples were then analyzed by flow cytometry to determine the percentages of c-*erb*B-2-positive (AU-565) and c-*erb*B-2-negative (Hela) cells expressing red fluorescent protein in infected and uninfected mixed cell cultures. The results of this experiment presented in Fig. 13 demonstrate that the sCARfC6.5 targeting adapter provided a 2.7- to 3-fold increase of Ad gene transfer to AU-565 cells compared to Ad alone while simultaneously decreasing gene transfer to Hela cells 18- to 27-fold. These data indicate that Ad targeting mediated by sCARfC6.5 adapter protein could improve overall selectivity of Ad infection for c-*erb*B-2-expressing breast cancer cells up to 72-fold compared to CAR-positive Hela cells.

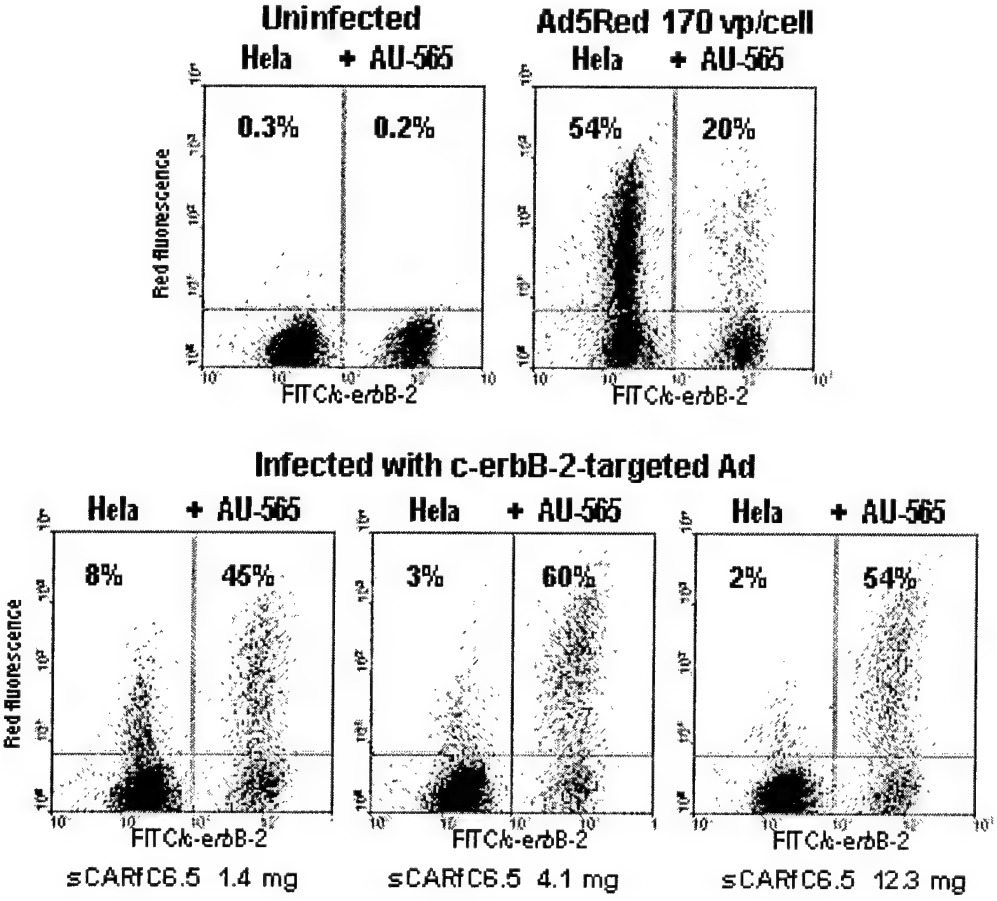

Fig. 13. Ad infection mediated by sCARfC6.5 adapter in a mixed cell culture. Ad5Red vector expressing red fluorescent protein was preincubated with 1.4, 4.1, or 12.3 μg of sCARfC6.5 (*c-erbB-2-targeted Ad*) or PBS (*Ad5Red*) and used to infect the cell monolayers formed by Hela and AU-565 cells at MOI of 170 vp/cell. Infected cells were harvested 24-h post-infection, stained for c-*erb*B-2 with Ab-2(9G6.10) mouse MAb using indirect immunofluorescence assay, and were then analysed by flow cytometry to determine the percentages of c-*erb*B-2-negative (*Hela*) and c-*erb*B-2-positive (*AU-565*) cells expressing red fluorescent protein relative to uninfected cells. The percentages of infected red fluorescent cells were calculated separately for c-*erb*B-2-negative and c-*erb*B-2-positive cell populations.

Task 3. To employ sCAR-ligand fusion proteins to accomplish targeted Ad infection in the context of animal model relevant to human breast cancer.

a. Estimate the potential of sCAR-ligand proteins to increase Ad-mediated gene delivery to human breast cancer xenografts established in the subcutaneous tissue of athymic nude mice.

i. Characterization of sCAR-ligand fusion proteins. Specificity of sCARfRGD and sCARfC6.5 fusion targeting proteins was first confirmed *in vitro* using an ELISA to evaluate binding to the Ad5 fiber knob protein and by a Western blot to confirm protein integrity. In each case the specific binding properties of fusion proteins were maintained (data not shown). To confirm the ability of sCARfRGD targeting protein to promote Ad vector infection of CAR-negative breast cancer cells via α_v-integrins, we used MDA-MB-435 breast adenocarcinoma cell line. To validate the ability of sCARfC6.5 targeting protein to mediate Ad infection of breast cancer cells via c-*erb*B-2 oncoprotein, we employed the derivative MDA-MB-435 cell line, MB-435.eB (kindly provided by Dr. Dihua Yu, The University of Texas M. D. Anderson Cancer Center, Houston, TX), stably transfected with human HER-2/*neu* gene and expresses c-*erb*B-2 at high levels (17, 18). In order to evaluate the ability of sCAR-ligand targeting proteins to direct Ad infection to breast cancer cells *in vitro*, an AdLuc vector carrying the luciferase reporter gene was used. This vector was complexed with either sCARfRGD or sCARfC6.5 protein by incubating virus with increasing amounts of the protein for 30 min at room temperature to prepare targeted viral complexes as we described previously. These complexes were then used to infect MDA-MB-435 or MB-435.eB cells, which do not express the native Ad receptor, CAR, but do express α_v-integrins, the secondary receptor, or c-*erb*B-2 oncoprotein respectively. As shown in Fig. 14, targeted AdLuc vectors were able to achieve enhanced, CAR-independent infection of these cells, with marked increase in gene delivery.

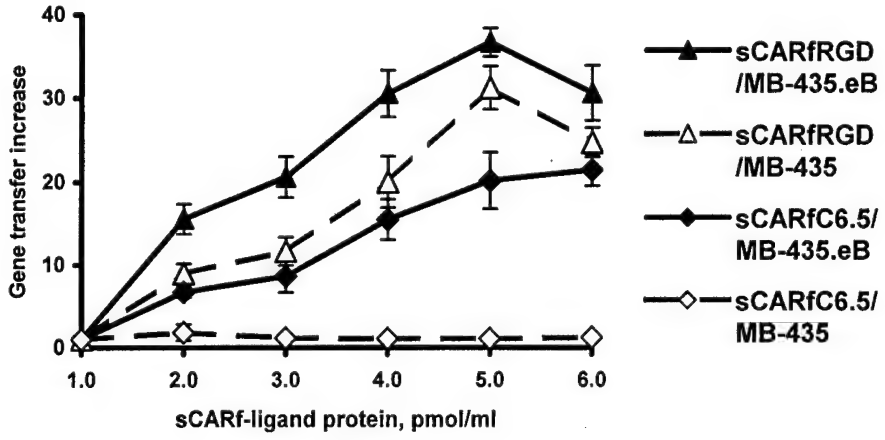

Fig. 14. sCAR-ligand-mediated Ad infection of breast cancer cells. AdLuc vector was complexed with sCARfRGD or sCARfC6.5 targeting proteins at varying concentrations to form integrin-targeted or c-*erb*B-2-targeted viral complexes, respectively. Monolayers of MDA-MB-435 or MB-435.eB cells were infected with targeted or Ad alone at a MOI of 100 vp/cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was measured. Results are presented as gene transfer indexes that were calculated as the ratio of luciferase activities detected in the cells infected with targeted Ad to luciferase activities detected in the cells infected with Ad alone. Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

The sCARfRGD targeting protein provided 31-fold and 36-fold increase of luciferase activity that was detected with Ad vector alone in MDA-MB-435 or MB-435.eB cells respectively. Virus complexes formed with c-*erb*B-2 -targeting sCARfC6.5 protein achieved 21-fold increase of luciferase activity compared to Ad vector alone when was used to infect MB-435.eB cells. Important that no enhancement of gene delivery was seen for AdLuc complexed with sCARfC6.5 to the parent MDA-MB-435 cells, which do not expresses c-*erb*B-2 oncoprotein. These results thus proved that a substantial increase in transduction efficiency of CAR-negative breast cancer cells could be achieved by targeting of Ad infection to α_v-integrins or c-*erb*B-2 oncoprotein, and further, that this mechanism could be employed to deliver genes to Ad-refractory breast cancer cell types *in vivo*.

ii. Analysis of Ad targeting to breast cancer cells at intratumoral injection *in vivo*. In keeping with our principal goal of *in vivo* targeting, we next evaluated the tumor targeting potential of Ad complexes formed using sCAR-ligand proteins following intratumoral injection in a murine model of human breast cancer. To evaluate Ad targeting properties of sCAR-ligand fusion proteins after direct intratumoral administration in a murine model of human breast cancer xenografts we compared gene transfer mediated by AdLuc vector alone to the levels of gene transfer achieved by AdLuc complexed with varying amounts of sCARfRGD protein. Three separate experiments were performed. Either Ad vector alone (10^9 v.p.) or the same viral dose of Ad/sCARfRGD complex was administered by direct multiple injections into subcutaneous tumor nodules formed by either MB-435.eB or MDA-MB-435 cells in NIH III nude mice (five mice per group). Two days later the mice were killed, tumor nodules and livers were harvested and analyzed for luciferase activity. For each analysis the entire tumor was snap-frozen, ground using a mortar and pestle, then cells were lysed in lysis buffer and luciferase activity in the supernatant was measured using a commercial kit (Promega) and a Berthold luminometer. A representative experiment is shown in Fig. 15.

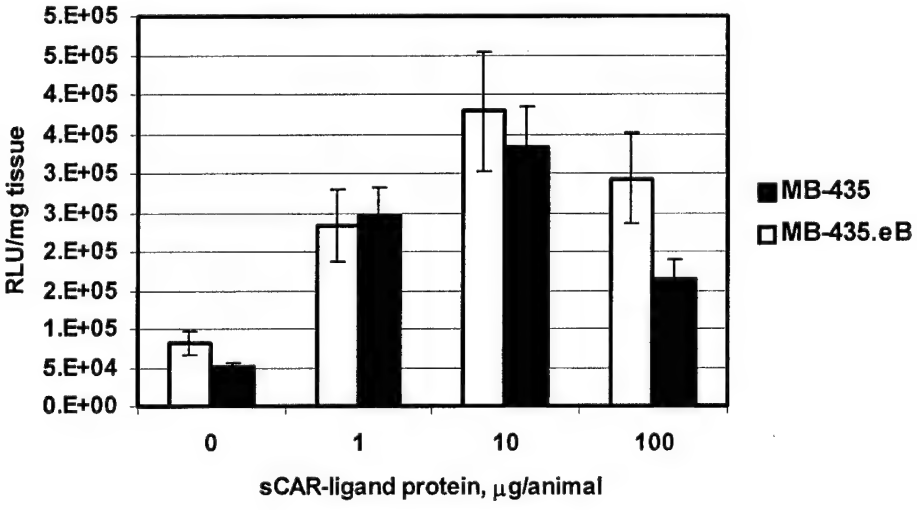

Fig. 15. sCAR-ligand-mediated Ad gene transfer after intratumoral injection. AdLuc was complexed with indicated amounts of sCARfRGD protein to form Ad/sCARfRGD targeted complexes. Either Ad vector alone or Ad/sCARfRGD complexes (10^9 v.p.) were administered by multiple injections into subcutaneous tumor nodules formed by either MB-435.eB or MDA-MB-435 cells in NIH III nude mice. Two days later the mice were killed, tumor nodules were harvested and luciferase activity was quantified. Data are means + SD of five mice per group.

Data are presented as relative light units (RLU) per milligram of tissue in the lysates. Analysis of luciferase activity revealed a significant difference in the levels of luciferase expression between AdLuc vector alone and Ad/sCARfRGD viral complexes. Notably, the levels of gene

transfer enhancements were different depending on the dose of sCARfRGD targeting protein used to form viral complexes. For both MB-435.eB and MDA-MB-435 tumor xenografts, the highest level of gene transfer was seen at 10 μg of sCARfRGD targeting protein per animal. Over three experiments mean luciferase expression in MDA-MB-435 tumor xenografts was 2.8-, 4.6-, and 3.6-fold higher for Ad/sCARfRGD (at the dose of 1, 10, 100 μg respectively), whereas luciferase activity in MB-435.eB tumors was 4.9, 6.7-, and 3.3-fold higher compared to Ad alone. The levels of luciferase activity in liver were very low with both Ad alone and Ad/sCARfRGD viral complexes and were not significantly different (data not shown). Thus, these data provide the evidence that Ad targeting to cellular integrins mediated by sCAR-ligand proteins can overcome CAR deficiency on breast cancer cell types and lead to enhanced tumor transduction and gene delivery in the context of intratumoral vector administration.

b. Characterize the tumor/normal organs distribution of ^{125}I-labeled sCAR-ligand fusion proteins administered by systemic injection into a tail vein of nude mice bearing a breast cancer xenografts.

To determine the biodistribution of sCARfRGD and sCARfC6.5 fusion proteins after systemic vascular administration in a murine model of human breast cancer, each protein was labeled with ^{125}I (3×10^5 cpm/g) in Iodo-Gencoated tubes (Pierce) as described previously (19). First, we choose to test radiolabeled proteins for their ability to bind to the cellular α_v-integrins or c-*erb*B-2 oncoprotein *in vitro*. To this end, MB-435.eB cells were incubated with either ^{125}I-sCARfRGD or ^{125}I-sCARfC6.5 at 37 or 4°C for 1 h. Unbound radioactivity was washed off, then the radioactivity associated with the cell surface was released by incubating the cells with 50 mM glycine, 100 mM NaCl, pH 2.5, for 15 min [at 37 or 4°C]. Cells were washed and trypsin–EDTA was added to detach the cells and determine the cell-associated radioactivity. Unfortunately, no specific cell-binding of radiolabeled proteins was seen above background level to MB-435.eB cells suggesting that the radiolabeling procedure had disturbed the receptor-binding specificities of sCAR-ligand fusion proteins (not shown). In order to confirm that radiolabeling has negatively affected the receptor-binding properties of sCAR-ligand proteins we used labeled and unlabeled sCARfRGD and sCARfC6.5 proteins to test their Ad targeting abilities as described in a. i). Fig. 16. shows the comparison of Ad gene transfer to MB-435.eB cells mediated by sCARfC6.5 compare to both radiolabeled sCAR-ligand proteins.

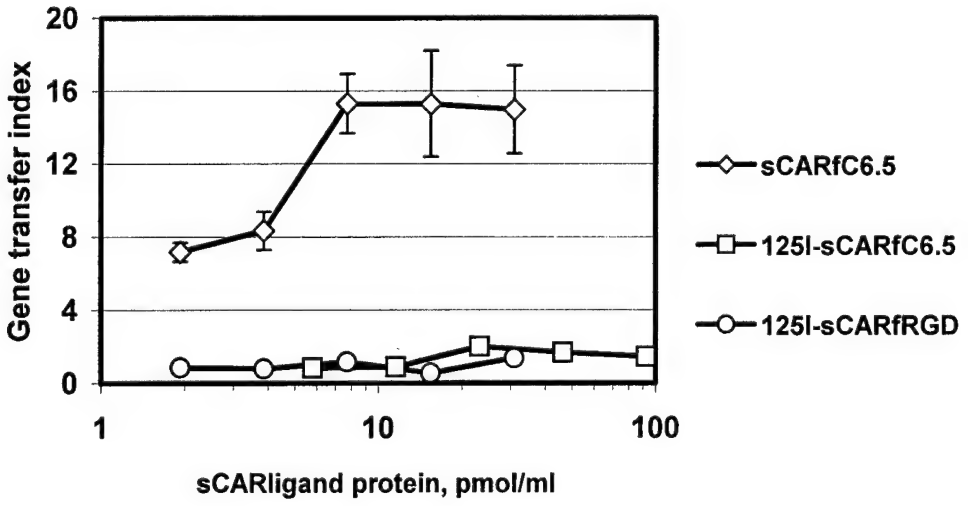

Fig. 16. Effect of sCAR-ligand protein radiolabeling on Ad targeting to MB-435.eB cells. AdLuc vector was complexed with sCARfC6.5, ^{125}I-labeled sCARfRGD, or ^{125}I-labeled sCARfC6.5 proteins at varying concentrations. Monolayers of MB-435.eB cells were incubated with Ad

alone or Ad/sCAR-ligand viral complexes at a MOI of 100 vp/cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was measured. Results are presented as gene transfer indexes that were calculated as the ratio of luciferase activities detected in the cells infected with Ad/sCAR-ligand complexes to luciferase activities detected in the cells infected with Ad alone. Data are means + SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

As could be seen from this figure, Ad targeting properties of radiolabeled ^{125}I-sCARfRGD and ^{125}I-sCARfC6.5 proteins were indeed disturbed, thus, precluding their use in biodistribution studies *in vivo*.

c. *Perform Ad targeting experiments to assess the ability of sCAR-ligand proteins mediate Ad gene delivery to tumor in vivo at systemic injection in a mouse model of human breast cancer.*

The ability of the sCARfRGD an sCARfC6.5 targeting proteins to direct Ad vector to the tumor was assessed using a murine model of human breast tumor xenografts created by injecting 10^7 breast adenocarcinoma cells subcutaneously into each flank of NIH III Nude mouse. Tumors were allowed to grow for two weeks or until tumor nodule is at least 100 mm^3 and animals were randomized by tumor size to form experimental and control groups (n = 5-7 mice/group). Aliquots of AdLuc vector (5×10^9 v.p.) were complexed with 10 μg of sCARf-ligand fusion protein (or incubated with diluent as a control) for 30 min at room temperature and then injected into the tail vein of tumor-bearing mice. Two days later, mice were sacrificed, tumor nodules and livers were harvested and luciferase activities per milligram tissue were determined. Luciferase expression in tumors and organs was variable between individual animals within experimental and control groups. Comparisons between groups were made using Student's t test, and significance was accepted at $P < 0.05$.

In order to illustrate the utility of Ad tropism modification approach based on the use of bispecific sCAR-ligand proteins we employed sCARfRGD fusion to assess systemic tumor targeting via integrin pathway. The results presented in Fig. 17 demonstrate Ad vector targeting to tumor xenografts established using MDA-MB-435 breast cancer cells.

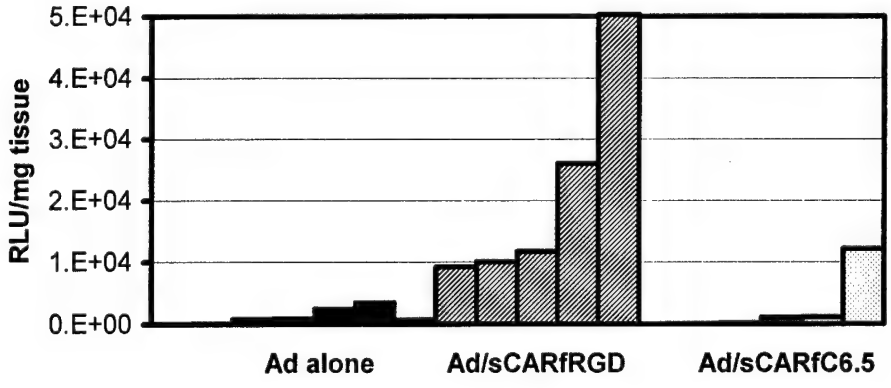

Fig. 17. The sCARfRGD targeting protein increases gene transfer to MB-435 tumor xenografts. NIH III nude mice bearing the MB-435 breast tumor xenografts were injected via tail vein with 5 x 10^9 v.p. of AdLuc vector alone or Adluc preincubated with either sCARfRGD or sCARfC6.5 targeting protein. Two days later the mice were killed, tumor nodules were harvested and luciferase activities were quantified. Raw data for each individual animal are presented.

As we showed previously these cells do not express the native Ad receptor, CAR, but do express integrins, the secondary viral receptor. Retargeted AdLuc vector complexed with sCARfRGD protein was able to achieve enhanced, CAR-independent systemic gene delivery to MB-435 tumor xenografts with mean increase in luciferase activity over three independent experiments of 13-fold compared to AdLuc alone (P = 0.035). To validate the specificity of sCARfRGD-mediated systemic Ad gene delivery via tumor cell integrins we compared its effect with sCARfC6.5, c-*erb*B-2-targeting protein, employed as a negative control (Fig. 17). Owing to the fact that MDA-MB-435 cells do not express c-*erb*B-2 oncoprotein, we did not expect sCARfC6.5 to affect Ad-mediated gene delivery (untargeted Ad) in this breast cancer animal model. When compared to the untargeted Ad vector, sCARfRGD-mediated targeting showed a substantial increase in tumor luciferase expression (mean increase over three experiments 7-fold, P = 0.043). Important, the difference between untargeted AdLuc complexed with sCARfC6.5 and AdLuc vector alone was not statistically significant (P = 0.3) thereby corroborating the specificity of Ad tumor targeting mediated by sCARfRGD *in vivo*.

To further demonstrate the ability of sCAR-ligand proteins to accomplish tumor-specific Ad-mediated gene delivery at systemic vascular administration we tested the effect of sCARfC6.5, c-*erb*B-2-targeting protein, *in vivo*. To this end, we employed MB-435.eB breast cancer cells overexpressing the c-*erb*B-2 oncoprotein (35-37), a molecule of established therapeutic relevance in breast cancer. As illustrated in Fig. 18 the use of sCARfC6.5 targeting protein provided significant augmentation of Ad-mediated gene transfer to subcutaneous MB-435.eB tumor xenografts compared to AdLuc alone (mean increase over three experiments 23-fold, P = 0.028). To validate the specificity of sCARfC6.5-mediated c-*erb*B-2 targeting *in vivo* we used sCARf fusion protein lacking C6.5 scFv ligand. As was expected tumor gene transfer achieved by untargeted Ad vector complexed with control sCARf protein was not significantly different compared to the level of luciferase expression demonstrated by Ad alone (P = 0.35). This experiment showed that only sCARfC6.5-mediated Ad targeting achieved enhanced transgene expression in the tumor in comparison to mice that received AdLuc alone or untargeted Ad complexed with sCARf (Fig. 18).

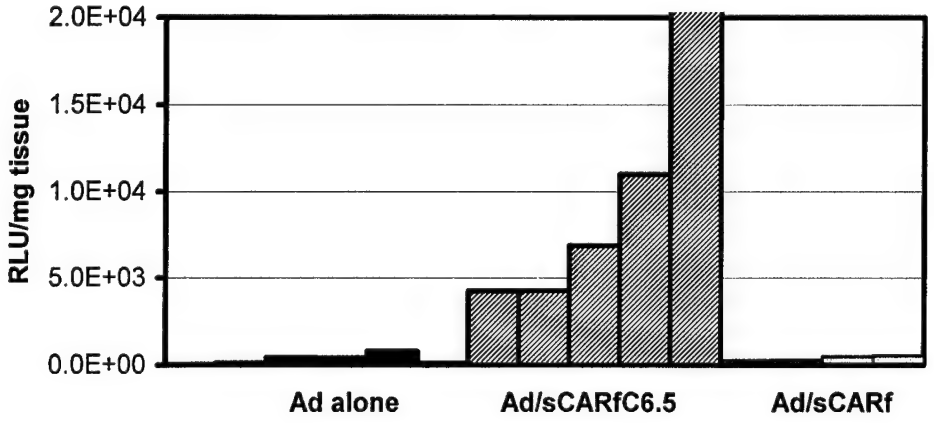

Fig. 18. The sCARfC6.5 targeting protein increases gene transfer to MB-435.eB tumor xenografts. NIH III nude mice bearing the MB-435.eB breast tumor xenografts were injected via tail vein with 5 x 10^9 v.p. of AdLuc vector alone or Adluc preincubated with either sCARfC6.5 or sCARf control protein. Two days later the mice were killed, tumor nodules were harvested and luciferase activities were quantified. Raw data for each individual animal are presented.

These data demonstrate that targeting of Ad infection to breast cancer cell types expressing c-*erb*B-2 by means of virus complexing with sCARfC6.5 protein provides marked

improvement of gene delivery to tumor xenografts *in vivo.* Thus, our studies established that retargeting of Ad infection to cancer cell types via cellular integrins and c-*erb*B-2 oncoprotein is able to provide tumor-specific enhancement of gene delivery to breast tumor xenografts via the systemic vascular administration route.

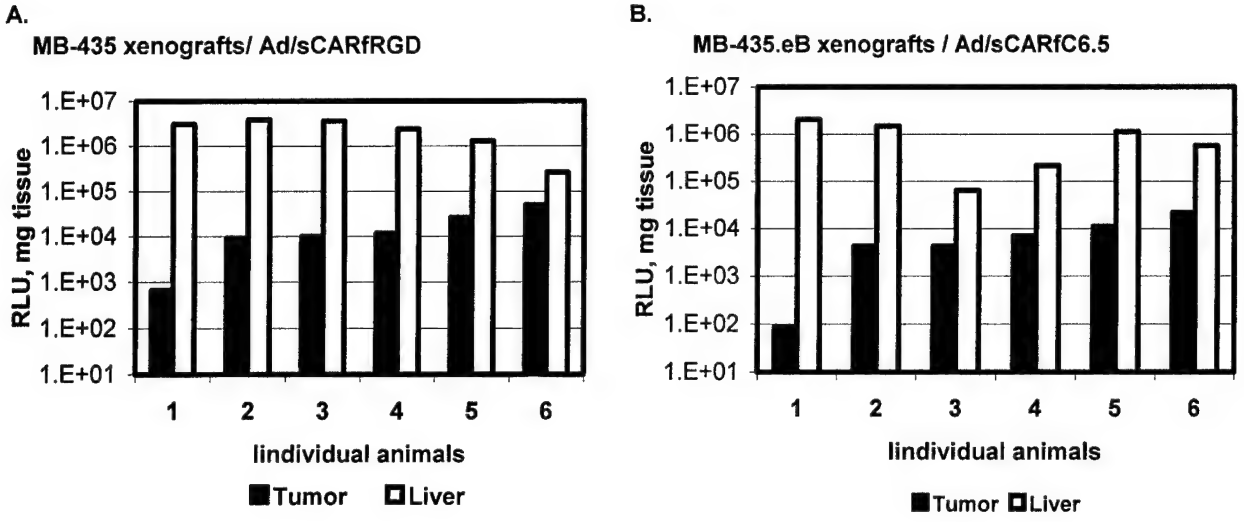

Fig. 19. Inhibition of Ad transduction of the liver by sCAR-ligand proteins. NIH III nude mice bearing tumor xenografts established using MDA-MB-435 or MB-435.eB breast adenocarcinoma cells were injected via tail vein with 5 x 10^9 v.p. of targeted Adluc complexes formed with either sCARfRGD (A) or sCARfC6.5 (B) or AdLuc vector alone. Two days later the mice were killed, tumor nodules and livers were harvested and luciferase activities were quantified. Raw data for each individual animal are presented.

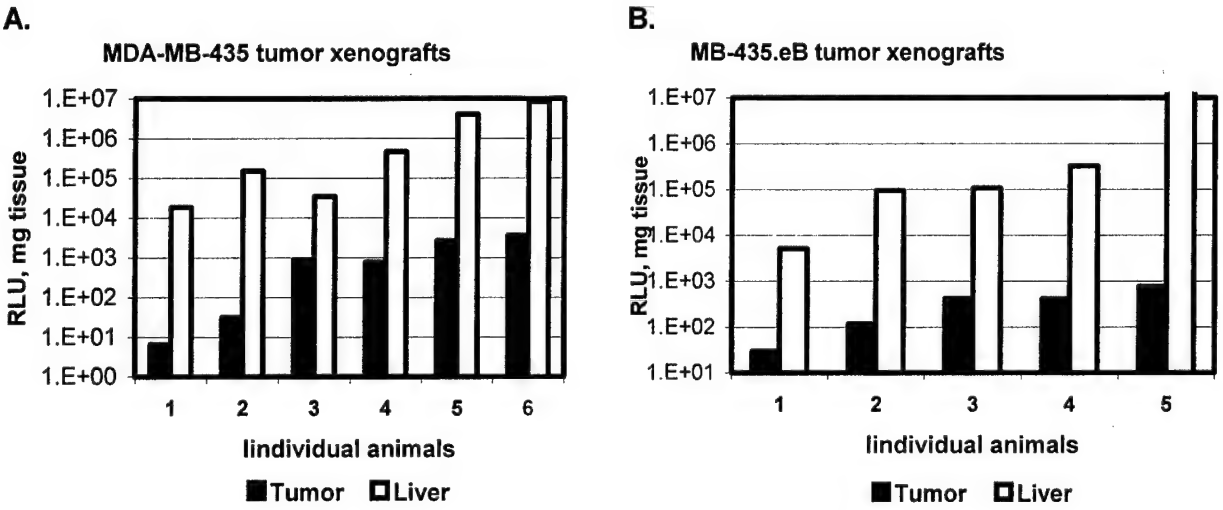

Fig. 20. Untargeted Ad vector increases both tumor and liver transduction. NIH III nude mice bearing tumor xenografts established using MDA-MB-435 (A) or MB-435.eB (B) breast adenocarcinoma cells were injected via tail vein with 5 x 10^9 v.p. AdLuc vector. Two days later the mice were killed, tumor nodules and livers were harvested and luciferase activities were quantified. Raw data for each individual animal are presented.

A key attribute of Ad vector targeting based on the use of bispecific protein adapters is that native Ad tropism could be ablated thereby decreasing viral transduction of nontarget organs and tissues. Fig. 19 shows the effect of our tumor targeting approach on liver transduction in mice that received either sCARfRGD- or sCARfC6.5-targeted Ad. There was an obvious trend

toward reduction of luciferase expression in the liver, which is most clearly seen in mice that demonstrated the highest tumor transduction. We believe that the observed reduction in liver transduction is a consequence of blocking of native CAR-dependent Ad tropism *in vivo* due to the complexing of the Ad fiber knob domain with sCAR-ligand targeting protein. In contrast, augmentation of Ad-mediated gene delivery to tumor xenografts in mice injected with AdLuc alone were most often associated with corresponding increase in liver transduction (Fig. 20) and could be explained by increase of effective systemic viral dose. The use of quantitative PCR to detect Ad vector DNA in tumor and liver tissues confirmed the results of gene transfer analysis (not shown).

Thus, our studies established that Ad infection mediated by bispecific adapter proteins was dependant upon unique cancer cells recognition properties of these reagents and their specificity for breast tumor xenografts and thus supported our hypothesis that tumor-targeted gene delivery could be achieved *in vivo* by the modification of Ad tropism. To our knowledge this is the first demonstration that an Ad vector can be selectively retargeted to tumor xenografts when administered via a peripheral vein.

KEY RESEARCH ACCOMPLISHMENTS

- Plasmid vector was constructed that contains recombinant gene encoding soluble CAR ectodomain, six-His purification tag, phage T4 fibritin polypeptide, hinge region and site for cloning of ligand sequences.
- Recombinant fusion proteins containing sCAR, fibritin trimerization domain and either RGD-4C or NGR peptide ligands were produced using baculovirus expression system and purified.
- Produced fusion proteins sCARf, sCARf-RGD4C, and sCARf-NGR were shown to form stable trimers and maintain designed composition.
- Trimeric sCAR-fusion proteins were shown to possess an augmented affinity to Ad fiber knob domain and increased ability to block CAR-mediated Ad infection compared to monomeric sCAR protein.
- Set of established breast cancer cell lines AU-565, SK-BR-3, BT-474, MCF-7, ZR-75-1, MB-468, and GI-101A was characterized for expression levels of CAR and α_V-integrins.
- We characterized recombinant sCARfNGR, sCARfRGD, and sCARfC6.5 targeting proteins for their ability to mediate Ad binding to breast cancer and endothelial cells expressing APN, α_V-integrins, and c-*erb*B-2 oncoprotein, respectively.
- In contrast to sCARfNGR, both sCARfRGD and sCARfC6.5 adapter proteins were capable of mediating viral infection via α_V-integrins and the c-*erb*B-2 oncoprotein providing up to 120-fold increases in Ad gene transfer to breast cancer cells compared to the sCARf control protein.
- We demonstrated that targeting of an Ad vector using the sCARfC6.5 adapter in mixed cell culture improves the selectivity of Ad infection for c-*erb*B-2-positive breast cancer cells up to 72-fold compared to CAR-positive Hela cells.
- We showed that Ad targeting to cellular integrins mediated by sCAR-ligand proteins can overcome CAR deficiency on breast cancer cell types and lead to enhanced tumor transduction and gene delivery in the context of intratumoral vector administration.
- Our studies established that retargeting of Ad infection to cancer cell types via cellular integrins and c-*erb*B-2 oncoprotein is able to provide tumor-specific enhancement of gene delivery to breast tumor xenografts via the systemic vascular administration route.

REPORTABLE OUTCOMES

The following manuscripts were published:

1. Wesseling, J. G., Bosma, P. J., Krasnykh, V., Kashentseva, E. A., Blackwell, J. L., Reynolds, P. N., Li, H., Parameshwar, M., Vickers, S. M., Jaffee, E. M., Huibregtse, K., Curiel, D. T., and Dmitriev, I. Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors, Gene Ther. *8:* 969-76., 2001.
2. Hemminki, A., Dmitriev, I., Liu, B., Desmond, R. A., Alemany, R., and Curiel, D. T. Targeting oncolytic adenoviral agents to the epidermal growth factor pathway with a secretory fusion molecule, Cancer Res. *61:* 6377-81, 2001.
3. Kashentseva, E. A., Seki, T., Curiel, D. T., and Dmitriev, I. P. Adenovirus targeting to c-*erb*B-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain, Cancer Res. *62:* 609-16., 2002.
4. Pereboev, A. V., Asiedu, C. K., Kawakami, Y., Dong, S. S., Blackwell, J. L., Kashentseva, E. A., Triozzi, P. L., Aldrich, W. A., Curiel, D. T., Thomas, J. M., and Dmitriev, I. P. Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells, Gene Ther. *9:* 1189-93., 2002.

The following meeting abstracts were published:

1. Kashentseva, E. A., Seki, T., Curiel, D. T., and Dmitriev, I. P. Utilization of soluble CAR-ligand proteins for adenovirus targeting. *In:* Fourth Annual Meeting of the American Society of Gene Therapy, Seattle, WA, USA, May 30 - June 3 2001. Published In: Molecular Ther., V. 3, N. 5, May 2001.
2. Dmitriev, I. P., Kashentseva, E. A., and Curiel, D. T. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody using recombinant adapter protein or genetic modification of viral capsid. *In:* Fifth Annual Meeting of the American Society of Gene Therapy, Boston, MA, USA, June 5-9 2002. Published In: Molecular Ther., V. 5, N. 5, pp. S203, May 2002.
3. Kashentseva, E. A. and Dmitriev, I. P. Adenovirus vector targeting to breast cancer by soluble adapter proteins. *In:* Era of Hope. The Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, USA, September 25-28 2002, pp. P32-5.

LIST OF PERSONNEL

1. DMITRIEV, IGOR P., Ph.D. – PI.
2. KASHENTSEVA, ELENA A. – Technical assistant.

CONCLUSIONS

In conclusion, the work presented in this report completely addressed all 3 tasks outlined in the approved Statement of Work. The utility of sCAR-ligand adapter proteins for Ad targeting to breast cancer cell types *in vitro* was characterized using virus-cell binding and gene transfer assays. The use of sCARfRGD and sCARfC6.5 targeting adapters allowed CAR-independent Ad binding and infection of breast cancer cells via α_V-integrins and the c-*erb*B-2 oncoprotein, respectively. It was shown that sCARfRGD and sCARfC6.5 adapter proteins were capable of providing up to 120-fold increases in Ad gene transfer to breast cancer cells compared to sCARf control protein (5). To illustrate the targeting potential of sCAR-ligand approach *in vitro* we carried out sCARfC6.5-mediated Ad infection in heterogeneous cell populations. We demonstrated that the use of the c-*erb*B-2-targeting adapter in mixed cell culture improved the selectivity of Ad infection for c-*erb*B-2-positive breast cancer cells up to 72-fold compared to CAR-positive Hela cells.

In order to extend the utility of this Ad targeting strategy we explored the ability of sCAR-ligand adapter to enhance an oncolytic effect of replication-competent vectors. We constructed a novel replication-deficient Ad expressing a secretory sCAR-EGF adapter capable of virus retargeting to EGFR, resulting in a more than 150-fold increase in gene transfer (20). Coinfection of this vector secreting the sCAR-EGF adapter with replication-competent Ad resulted in increased oncolysis *in vitro* and therapeutic benefit *in vivo*.

Further, we demonstrated that retargeting of Ad infection to cancer cell types via cellular integrins and c-*erb*B-2 oncoprotein is able to provide tumor-specific enhancement of gene delivery to breast tumor xenografts via the systemic vascular administration route. Our animal experiments established that Ad infection mediated by bispecific adapter proteins was dependant upon unique cancer cells recognition properties of these reagents and their specificity for breast tumor xenografts and thus supported our hypothesis that tumor-targeted gene delivery could be achieved *in vivo* by the modification of Ad tropism. To our knowledge this is the first demonstration that an Ad vector can be selectively retargeted to tumor xenografts when administered via a peripheral vein.

These results strongly suggest that Ad vector targeting using recombinant sCAR-ligand adapter proteins could augment the selectivity of Ad-mediated gene transfer for breast tumors. Our data cliarly indicate that the RGD motif and anti-c-*erb*B-2 scFv are promising ligands for targeted Ad vector delivery to breast cancer cell types. Thus, Ad targeting approaches employing sCAR-ligand adapters are expected to increase the efficiency of therapeutic gene delivery and decrease the toxicity of Ad vectors, which should improve the therapeutic index of cytotoxic gene therapy for carcinoma of the breast in clinical trials.

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Adenovirus Targeting to c-*erb*B-2 Oncoprotein by Single-Chain Antibody Fused to Trimeric Form of Adenovirus Receptor Ectodomain[1]

Elena A. Kashentseva, Toshiro Seki, David T. Curiel, and Igor P. Dmitriev[2]

Division of Human Gene Therapy [E. A. K., T. S., D. T. C., I. P. D.], Departments of Medicine, Pathology, and Surgery [D. T. C.], and Gene Therapy Center [D. T. C.], University of Alabama at Birmingham, Birmingham, Alabama 35294-3300

ABSTRACT

The use of adenovirus (Ad) vectors for cancer gene therapy applications is currently limited by several factors, including broad Ad tropism associated with the widespread expression of *c*oxsackievirus and *a*denovirus *r*eceptor (CAR) in normal human tissues, as well as limited levels of CAR in tumor cells. To target Ad to relevant cell types, we have proposed using soluble CAR (sCAR) ectodomain fused with a ligand to block CAR-dependent native tropism and to simultaneously achieve infection through a novel receptor overexpressed in target cells. To confer Ad targeting capability on cancer cells expressing the c-*erb*B-2/HER-2/neu oncogene, we engineered a bispecific adapter protein, sCARfC6.5, that consisted of sCAR, phage T4 fibritin polypeptide, and C6.5 single-chain fragment variable (scFv) against c-*erb*B-2 oncoprotein. Incorporation of fibritin polypeptide provided trimerization of sCAR fusion proteins that, compared with monomeric sCAR protein, resulted in augmented affinity to Ad fiber knob domain and in increased ability to block CAR-dependent Ad infection. We demonstrated that sCARfC6.5 protein binds to cellular c-*erb*B-2 oncoprotein and mediates efficient Ad targeting via a CAR-independent pathway. As illustrated in cancer cell lines that overexpress c-*erb*B-2, targeted Ad, complexed with sCARfC6.5 adapter protein, provided from 1.5- to 17-fold enhancement of gene transfer compared with Ad alone and up to 130-fold increase in comparison with untargeted Ad complexed with sCARf control protein. The use of recombinant trimeric sCAR-scFv adapter proteins may augment Ad vector potency for targeting cancer cell types.

INTRODUCTION

Ad[3] represents a large family of nonenveloped viruses (1). Human Ad includes 47 known viral serotypes grouped into six distinct subgroups, A to F. Most of the studies on the mechanism of Ad infection have concluded that receptor recognition is one of the key factors that determines cell tropism (2, 3). In this regard, the initial steps of Ad infection involve at least two sequential virus-cell interactions, each mediated by a specific viral capsid protein. Ad infection is initiated by the binding of globular knob domain of trimeric fiber protein to a host cell primary receptor (4, 5). Subsequent interaction of the penton base with α_v integrins mediates virion internalization via receptor-mediated endocytosis (6). Fiber receptor for Ad subgroups A, C, D, E, and F has been identified as the CAR (7–9). CAR is an integral membrane protein consisting of two extracellular immunoglobulin-like D1 and D2 domains, a transmembrane region, and a COOH-terminal cytoplasmic domain (8, 10). The extracellular domain of CAR is sufficient for virus attachment and infection (11, 12), whereas both transmembrane and intracellular regions appear to be dispensable for these functions (13). Both structural analysis of fiber knob complexed with CAR D1 domain (14) and knob mutagenesis studies (15) revealed that amino acid residues responsible for CAR binding are located on lateral surfaces formed by the interface of two adjacent knob monomers. These data suggest an avidity mechanism when three CAR molecules could simultaneously bind per one fiber knob trimer, which was recently supported by kinetic analysis of Ad2 knob binding to the CAR D1 domain (16).

Well-characterized Ad serotypes 2 and 5 from subgroup C are predominantly used as vectors for *in vitro* and *in vivo* gene delivery (17), because of high infection efficiency in a variety of human cell types and tissues. However, this broad viral tropism is disadvantageous for gene delivery to cancer cell types refractory to Ad infection because of the absence or low levels of CAR expression (18–21). This limitation could be solved by Ad targeting via a nonnative viral receptor (22, 23). Several strategies have been tested in an effort to target Ad via CAR-independent pathways (24) including chemical conjugation or genetic modification of viral capsid proteins to incorporate targeting ligands and the use of bispecific adapter molecules to provide indirect virus linkage with the cell-surface receptors. The technical achievement of Ad targeting via adapter molecules has been approached by a variety of methods. Bispecific conjugates of antibodies or their Fab fragments were used to achieve linkage between target receptor and v.p. by means of specific recognition through either a fiber knob domain or penton base (reviewed in Refs. 17, 22, 23). Further refinement of this strategy has been accomplished by the engineering of recombinant proteins consisting of an anti-knob scFv fused with human EGF (25) or a scFv against EGFR (26). The original concept of employment of fusion proteins comprising a soluble viral receptor and targeting ligand was proposed for retrovirus targeting to specific cell types (27). Applying this strategy to Ad targeting, we have developed an approach based on the use of sCAR ectodomain fused with EGF, achieving simultaneously the blocking of virus-CAR interaction and the redirection of Ad to cells overexpressing EGFR (28, 29). A similar approach was successfully applied to target Ad to high-affinity Fcγ receptor I-positive human monocytic cells (30). The use of recombinant adapter molecules eliminates chemical conjugation and provides a high degree of flexibility for ligand substitution and, consequently, expands the targeting capabilities of Ad vectors.

We hypothesized that the predicted 3:1 stoichiometry of CAR-knob binding could provide high-affinity linkage of trimeric sCAR-ligand proteins to v.p. and, thereby, promote the ligand-mediated binding to target receptors. In this study, we describe a novel approach of Ad transductional targeting to cancer cell types expressing c-*erb*B-2 oncoprotein by means of a recombinant protein adapter. The gene known as c-*erb*B-2/HER-2/*neu*, encoding a member of the *erb*B family of growth factor receptors, is most frequently altered in human cancer and was shown overexpressed in a number of malignancies including tumors that arise in the breast and ovary (31, 32). We engineered a bispecific protein, sCARfC6.5, featuring a unique trimeric design and consisting of sCAR fused with phage T4 fibritin polypeptide and C6.5 scFv against c-*erb*B-2. We have demonstrated that the sCARfC6.5 protein efficiently blocks Ad native tropism while simultaneously

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[2] To whom requests for reprints should be addressed, at the University of Alabama at Birmingham, WTI-620, 1824 Sixth Avenue South, Birmingham, AL 5294-3300. Phone: (205) 934-2326; Fax: (205) 975-7949; E-mail: idmitriev@gtp.ccc.uab.edu.

[3] The abbreviations used are: Ad, adenovirus; CAR, coxsackievirus and Ad receptor; FACS, fluorescent-activated cell sorting; sCAR, soluble CAR ectodomain; scFv, single-chain fragment variable; EGF, epidermal growth factor; EGFR, EGF receptor; MAb, monoclonal antibody; GFP, green fluorescent protein; v.p., viral particle(s); MOI, multiplicity/multiplicities of infection; FBS, fetal bovine serum.

mediating virus infection via an alternative CAR-independent pathway, which markedly enhances gene transfer efficiency to cell lines that overexpress c-*erb*B-2. Our data suggest that the use of this original approach may augment the potency of Ad vectors for cancer gene therapy.

MATERIALS AND METHODS

Cells and Media. The 293 human kidney cell line, transformed with Ad5 DNA, was purchased from Microbix (Toronto, Ontario, Canada). The human breast cancer cell lines MDA-MB-468, AU-565, SK-BR-3, BT-474, and MCF-7 and the ovarian cancer cell line SK-OV-3, established from adenocarcinomas of mammary gland and ovary, respectively, were from the American Type Culture Collection (Manassas, VA). All of the cell lines were maintained in recommended growth media supplied by Mediatech (Herndon, Va.) containing 10% FBS (HighClone, Logan, UT) and 2 mM glutamine at 37°C in a humidified atmosphere of 5% CO_2. Infection of the cells with Ad was carried out in the infection medium containing 2% FBS.

Enzymes. Restriction endonucleases, Klenow enzyme, T4 DNA ligase, and proteinase K were from either New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN).

Antibodies. Murine serum to baculovirus-produced human sCAR protein was generated at the University of Alabama at Birmingham Hybridoma Core Facility. The MAb RmcB (33) to human CAR were produced using hybridoma purchased from American Type Culture Collection and kindly provided by J. T. Douglas (University of Alabama at Birmingham). Penta·His MAbs were from Qiagen Inc. (Valencia, CA). Rabbit serum against phage T4 fibritin protein was kindly provided by V. Mesyanzhinov (Shemykin and Ovchinnicov Institute of Bioorganic Chemistry, Moscow, Russia). Mouse MAbs to the human c-*erb*B-2/HER-2/neu oncoprotein, Ab-2 (Clone9G6.10), were purchased from NeoMarkers Inc., (Fremont, CA). Normal mouse IgG1 were from OEM Concepts (Toms River, NJ). Goat antimouse and antirabbit IgG conjugated with alkaline phosphatase were from Sigma Chemical Co. (St Louis, MO) and Pierce (Rockford, IL), respectively. Streptavidin-alkaline phosphatase conjugate was from Bio-Rad Laboratories (Hercules, CA). Alexa 488-labeled goat antimouse IgG were from Molecular Probes (Eugene, OR).

Viruses. A recombinant Ad5 vector, AdLucGFP, containing double expression cassette consisting of firefly luciferase gene and *GFP* gene under the control of cytomegalovirus immediate early promoter in place of the E1 region of the Ad genome, was constructed as described by Seki *et al.* (34). Ad was propagated on 293 cells and purified by centrifugation in CsCl gradients by a standard protocol. The titers of physical v.p. and infectious v.p. were determined by using the methods of Maizel *et al.* (35) and Mittereder *et al.* (36), respectively.

Construction of Recombinant Plasmids. To generate the recombinant gene encoding the extracellular domain of human CAR followed by polypeptide sequence derived from bacteriophage T4 fibritin protein (37), PCR was used. Sense primer 5′-GTT GAA AGA TCT GGA TTA ACC AAT AAA ATA AAA GCT ATC GAA ACT GAT ATT GCA TCA G complementary to position 1240 of the fibritin gene was designed to introduce *Bgl*II restriction site into the amplified DNA sequence, and antisense primer 5′-TTG CGG CCC CAG CGG CCG CTG GTG ATA AAA AGG TAG complementary to position 16 of untranslated 3′-region was designed both to introduce *Not*I restriction site and to substitute a stop codon for an alanine (GCC) codon. The PCR fragment (238 bp) was digested with *Bgl*II and *Not*I, and a 214-bp DNA fragment encoding 71 COOH-terminal amino acids of fibritin M polypeptide (38) was purified. A *Bgl*II-*Not*I-fragment was ligated with *Bam*HI- and *Not*I-digested plasmid pFBshCAR-EGF (29) that contained the recombinant gene for the CAR ectodomain, His_6, short linker, and human EGF to substitute EGF for the fibritin sequence. This plasmid, designated pFBsCARfibritin, was then cleaved with *Not*I and ligated with oligonucleotide duplex 5′-GGC CCA ACC GCA GCC AAA ACC TCA ACC CCA GCC ACA ACC TCA GCC CAA ACC TCA GCC TAA ACC GGT TTA AAC GGC C coding for a proline-rich hinge region that was derived from camel immunoglobulins and containing an *Age*I site followed by a stop codon. A plasmid clone that contained the DNA duplex in the correct orientation was selected by sequencing and was designated pFBsCARfCh. The resultant plasmid was then used as a vector to generate the recombinant baculovirus using the Bac-to-Bac baculovirus construction system (Life Technologies, Inc., Grand Island, N.Y.) to express sCARf protein. Then, oligonucleotides 5′-CCG GGA GCT CTG CGC TAG CT and 5′-CCG GAG CTA GCG CAG AGC TC, designed to contain *Sac*I and *Nhe*I restriction sites and *Age*I-compatible cohesive 5′-ends, were annealed to form duplex DNA and ligated to *Age*I-digested pFBsCARfCh. Plasmid clones were sequenced, and the plasmid containing the DNA duplex in the correct orientation was designated pFBsCARfChSN. DNA sequence coding for C6.5 scFv against c-*erb*B-2 was PCR amplified from cDNA (provided by J. D. Marks, Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA) using primers 5′-AGG AAA CCG GTG GTC TAG ATC AGG TGC AGC and 5′-AGT ATC TAG AGG GAA CTA GTA CGG TCA GCT TGG TCC CTC, which were designed to introduce *Xba*I and *Spe*I restriction sites, respectively. The PCR product was digested with *Xba*I and *Spe*I, and a purified 769-bp DNA fragment was cloned into *Spe*I-cleaved pFastBacHTa (Life Technologies, Inc.), which resulted in plasmid pFB6hC6.5. Then, plasmid pFBsCARfChSN was digested with *Sac*I and *Nhe*I and ligated with 775-bp *Sac*I-*Spe*I-fragment DNA, coding for C6.5 scFv isolated from pFB6hC6.5. The constructed plasmid, containing recombinant gene encoding sCAR, His_6, short linker, fibritin polypeptide, hinge region, and C6.5 scFv, was sequenced to confirm the correct DNA structure. The resultant plasmid, designated pFBsCARfC6.5, was then used to generate the recombinant baculovirus using the Bac-to-Bac system.

Expression, Purification, and Biotinylation of the Fusion Proteins. The fusion proteins, sCARf and sCARfC6.5, comprised of sCAR-His_6-fibritin and sCAR-His_6-fibritin-hinge-C6.5scFv polypeptide sequences, respectively, were expressed in High Five cells (Invitrogen, Carlsbad, CA) that were infected with recombinant baculoviruses. Recombinant His_6-tagged proteins were purified from dialyzed culture medium by immobilized metal-ion-affinity chromatography on Ni-nitrilotriacetic acid (Ni-NTA)-Sepharose (Qiagen Inc.) as described previously (29). Protein concentrations were determined by the BCA-200 protein assay kit using bovine gamma globulin as the standard (Pierce). Purified sCARf and previously produced sCAR-His_6 (29) proteins were biotinylated using EZ-Link SulfoNHS-LS-Biotinylation kit (Pierce). The degree of biotin-protein incorporation [determined using HABA method (Pierce)] was 0.6 biotin per molecule of sCAR-His_6 monomeric protein and 0.5 biotin per trimeric molecule of sCARf protein.

Protein Electrophoresis and Western Blot. To determine whether the recombinant sCARf and sCARfC6.5 fusion proteins could form trimers, they were analyzed by SDS-PAGE. Purified proteins were either boiled in Laemmli loading buffer prior to electrophoresis to denature proteins to monomers or loaded on the gel without denaturation. The trimeric or monomeric configurations of protein molecules were determined based on their mobilities in the gel. To analyze the composition of sCAR fusion proteins, we used Western blot. Samples of boiled sCARfC6.5 and sCARf proteins separated on 4–15% gradient SDS-PAGE were transferred to polyvinylidene difluoride membrane and probed with murine anti-sCAR serum, Penta·His MAb, or rabbit antifibritin serum. Bound IgG were detected with secondary alkaline phosphatase-conjugated antibodies.

ELISA. Solid-phase binding ELISA was performed as follows. Recombinant Ad5 knob protein, expressed in *Escherichia coli* as described previously (39), was diluted in 50 mM $NaHCO_3$ (pH 9.6) at a concentration of 1 μg/ml and was immobilized on Nunc-Maxisorp ELISA plate overnight. The wells were blocked with PBS [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), and 136 mM NaCl] containing 0.05% Tween 20 and 2% BSA and then were washed with PBS containing 0.05% Tween 20. Biotinylated sCAR-His_6 and sCARf proteins, diluted in blocking buffer to concentrations ranging from 0.01 to 25 pmol/ml, were added to the wells in 100-μl aliquots. After a 1-h incubation at room temperature, the wells were washed, and bound biotinylated proteins were detected by 45-min incubation with 1:1000 dilution of streptavidin-alkaline phosphatase conjugate (Bio-Rad). The plates were then developed using signal-producing reagent *p*-nitrophenyl phosphate (Sigma Chemical Co.). Plates were read in a microtiter plate reader, set at 405 nm; results are presented as mean absorbance ± SD.

Indirect Immunofluorescence. The analysis of cell lines for expression levels of CAR and c-*erb*B-2 oncoprotein was performed by indirect immunofluorescence assay using flow cytometry as follows. Aliquots (100 μl) of cells, resuspended in FACS buffer [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), 136 mM NaCl, 1% BSA, and 0.1% NaN_3] at a concentration of 2×10^6 cells/ml were incubated with either RmcB (anti-CAR) or Ab-2 (anti-c-*erb*B-2) MAb at

a concentration of 5 μg/ml for 1 h at 4°C. An isotype-matched normal mouse IgG1 was used as a negative control. Cells were washed with FACS buffer by centrifugation and then were incubated with secondary Alexa 488-labeled goat antimouse antibody (Molecular Probes) at a concentration of 5 μg/ml for 1 h at 4°C. Cells were washed with FACS buffer prior to flow cytometry analysis. To validate that C6.5 scFv, incorporated in the context of sCARfC6.5 protein, are able to bind to cellular c-*erb*B-2, cells were incubated first with sCARfC6.5 or with sCARf protein as a negative control at a concentration of 10 μg/ml. After a 1-h incubation, cells were washed and incubated with primary RmcB MAb and then with secondary Alexa 488-labeled antibody as described above. Cell samples (10^4 cells/sample) were analyzed by flow cytometry performed at the University of Alabama at Birmingham FACS Core Facility. Data were expressed as the geometric mean fluorescence intensity of the entire gated population. The positive cell population was determined by gating the right-hand tail of the distribution of the negative control sample for each cell line at 1%.

Gene Transfer Assay. The assay of Ad-mediated gene transfer to the cells was performed as follows. Aliquots (3 μl) of AdLucGFP vector were mixed with 6-μl aliquots of sCARf or sCARfC6.5 protein dilutions ranging from 0.2 to 53 pmol or of PBS [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), and 136 mM NaCl] for 15 min at room temperature. The virus-sCARprotein-containing complexes were diluted to 1 ml with infection medium containing 2% FBS, and 200-μl aliquots were then added to the cell monolayers [grown in a 24-well plate (3–5 × 10^5 cells/well) at MOI of 100 v.p./cell] and were incubated for 30 min at room temperature to allow virus internalization. Then, infection medium was aspirated, the cells were washed with PBS, and the cells were incubated in a growth medium containing 10% FBS at 37°C to allow expression of the reporter genes. Forty-six h postinfection, cells were lysed and luciferase activity was analyzed by using the Promega (Madison, WI) luciferase assay system and a Berthold (Gaithersburg, MD) luminometer. For inhibition of the Ad infection of 293 cells AdLucGFP vector was mixed with sCARf or sCARfC6.5 protein dilutions (1.1–30 pmol), or with sCAR-His_6 protein dilutions (3–230 pmol), or with PBS for 15 min at room temperature. Monolayers of 293 cells were exposed to the virus-sCARprotein-containing complexes at MOI of 13 v.p./cell for 30 min and then were incubated for an additional 20 h at 37°C to allow expression of luciferase gene prior to analysis.

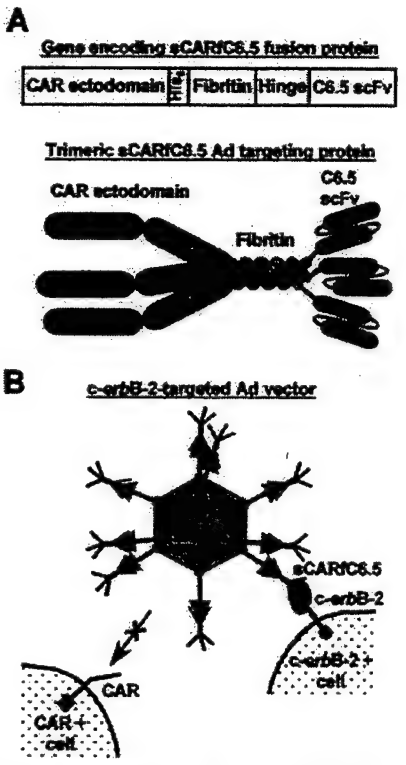

Fig. 1. *A*, design of sCARfC6.5 fusion protein. The recombinant fusion gene encoding the human CAR ectodomain, His_6, polypeptide derived from phage T4 fibritin protein (*Fibritin*); proline-rich hinge region (*Hinge*), and C6.5 scFv to c-*erb*B-2 oncoprotein was constructed in a baculovirus expression vector. The sequence encoding fibritin polypeptide was introduced into the design to achieve sCARfC6.5 protein trimerization. Recombinant gene was expressed in baculovirus-infected insect cells and secreted sCARfC6.5 protein was purified. *B*, use of sCARfC6.5 protein for Ad targeting. Engineered bispecific sCARfC6.5 fusion protein serves as an adapter between Ad and cells expressing c-*erb*B-2 oncoprotein. Presence of three sCAR domains in the context of trimerized adapter molecule potentially provides high-affinity viral linkage because of the trivalent stoichiometry of CAR-knob binding. The use of trimeric sCARfC6.5 adapter protein might, therefore, allow efficient blocking of Ad infection of CAR-bearing cells. C6.5 scFv targeting moiety of virus-bound adapter protein mediates recognition of c-*erb*B-2-positive cells, thereby providing novel target-specific Ad tropism.

RESULTS

Design and Generation of sCAR Fusion Proteins. To exploit the trivalent nature of CAR-knob interaction for the purposes of Ad targeting, we engineered a recombinant adapter protein consisting of soluble CAR in fusion with a trimerization sequence and a targeting ligand (Fig. 1*A*). We hypothesized that the predicted 3:1 stoichiometry of CAR-knob binding could provide high-affinity linkage of trimeric sCAR-ligand adapter proteins to virus and thereby block CAR-dependent Ad infection. Our goal was to generate a trimeric sCAR-ligand protein capable of efficiently blocking Ad native tropism while providing a novel target-selective tropism to c-*erb*B-2-positive cells (Fig. 1*B*). Because of the absence of a specific cognate ligand for c-*erb*B-2 oncoprotein, we chose C6.5 scFv as a targeting moiety that binds to the extracellular domain of this tumor antigen (40).

The gene encoding the extracellular part of human CAR including secretion signal, six-histidine tag (His_6), flexible linker, trimerization domain, hinge region, and C6.5 scFv was designed to produce sCARfC6.5 targeting protein. To achieve trimerization of the sCAR fusion molecule, we used a polypeptide derived from bacteriophage T4 fibritin protein, which is known to form highly stable homotrimers (37). Genetically engineered fibritin M polypeptide (38), containing 71 amino acids corresponding to the last α-helical coiled-coil segment and the complete COOH-terminal domain of phage T4 fibritin protein, was used. A proline-rich hinge region (20 amino acids) derived from camel antibodies served as a junction between the fibritin polypeptide and downstream scFv. The gene encoding sCAR, His_6, linker, fibritin M, and hinge region was used to produce sCARf control protein. The constructed genes for sCARf and sCARfC6.5 proteins were expressed in a baculovirus expression system that has already proved its utility for the expression of functional sCAR (9) and chimeric sCAR-EGF (29). Both of the fusion proteins were produced in a secreted soluble form after infection of High Five insect cells with generated recombinant baculoviruses. Secreted sCARf and sCARfC6.5 His_6-tagged proteins were purified and were analyzed for the presence of encoded polypeptide sequences and trimerization.

Characterization of Recombinant sCAR Fusion Proteins. The polypeptide composition of produced fusion proteins was characterized by Western blot analysis. Detection of denatured electrophoretically resolved sCARf and sCARfC6.5 proteins using specific antibodies revealed the presence of the CAR ectodomain, His_6 tag, and fibritin sequences in the context of both of the fusion proteins (Fig. 2*A*). The incorporation of scFv sequence in sCARfC6.5 protein was confirmed by the shift of its electrophoretic mobility compared with sCARf, indicating the predicted 27 kDa increase of molecular mass. The presence of additional minor bands in the sample of sCARfC6.5 protein was likely the result of incomplete translation of sCARfC6.5 mRNA. To determine whether the recombinant secreted sCARf and sCARfC6.5 proteins form trimers, these proteins were analyzed by SDS-PAGE. Electrophoresis of denatured protein samples showed the presence of major bands with molecular masses close to 36 and 63 kDa as expected for monomeric forms of sCARf and sCARfC6.5 molecules, respectively (Fig. 2*B*). Electrophoretic mobility of nonde-

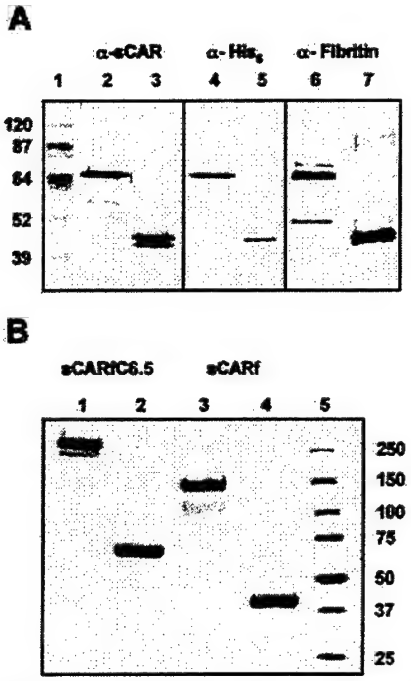

Fig. 2. Characterization of sCAR fusion proteins. *A*, Western blot of sCAR fusion proteins. Samples of purified sCARfC6.5 (*Lanes 2, 4,* and *6*) and sCARf (*Lanes 3, 5,* and *7*) proteins were boiled in Laemmli loading sample buffer and separated on 4–15% gradient SDS-PAGE. Electrophoretically resolved proteins were transferred to polyvinylidene difluoride membrane and probed with murine anti-sCAR serum (*Lanes 2* and *3*), Penta-His MAb (*Lanes 4* and *5*), or rabbit anti-fibritin serum (*Lanes 6* and *7*). Bound murine or rabbit IgG were detected with secondary alkaline phosphatase-conjugated goat antimouse or antirabbit antibodies, respectively. *Numbers on the left*, molecular masses of marker proteins (*Lane 1*) in kilodaltons. *B*, trimerization analysis of sCAR fusion proteins. Samples of sCARfC6.5 (*Lanes 1* and *2*) and sCARf (*Lanes 3* and *4*) proteins and molecular mass marker (*Lane 5*) were separated on 4–15% gradient SDS-PAGE. The samples in *Lanes 2* and *4* were boiled in Laemmli loading buffer to denature proteins to monomers, whereas proteins in *Lanes 1* and *3* were not denatured by boiling. Protein bands were visualized by GELCODE blue stain reagent. *Numbers on the right*, molecular masses of marker proteins in kilodaltons.

natured protein samples was greatly decreased compared with denatured proteins as was predicted for trimeric forms of sCARf and sCARfC6.5 molecules. This demonstrated that incorporation of fibritin polypeptide in the context of these recombinant fusion proteins results in efficient trimerization of both sCARfC6.5 and sCARf control protein. Thus, the analysis of sCARf and sCARfC6.5 proteins indicates that generated sCAR fusion proteins maintain both designed composition and stable trimeric conformation.

Analysis of sCAR Fusion Proteins Binding to Ad Fiber Knob. We characterized trimeric sCARf protein with respect to its ability to bind Ad fiber knob compared with monomeric sCAR-His_6 protein generated previously (29). The knob-binding affinities of sCARf and sCAR-His_6 proteins were compared by ELISA using immobilized Ad5 knob expressed in *E. coli* (39). Compared with monomeric sCAR-His_6 protein, the knob-binding affinity of trimeric sCARf protein was increased at least 20-fold in a range of tested concentrations from 0.1 to 5 pmol/ml (Fig. 3*A*). This result suggests that, compared with sCAR-His_6 monomer, trimeric sCARf protein possesses augmented ability to provide viral linkage by means of high-affinity binding to Ad fiber knob domain. Therefore, trimeric sCAR fusion proteins might offer improved blocking capability of CAR-dependent virus-cell attachment and viral infection.

To determine whether augmented binding to Ad fiber knob results in increased ability of trimeric sCAR proteins to block Ad infection, we performed an infection inhibition assay. AdLucGFP vector, expressing both luciferase and *GFP* reporter genes, was preincubated with either PBS or one of the sCAR-His_6, sCARf, or sCARfC6.5 proteins at varying concentrations and was used to infect 293 cells, which are known to express a high level of CAR. The ability of sCAR fusion proteins to block viral infection was assessed by sCAR protein dose-dependent impairment of Ad-mediated gene transfer as measured by luciferase activity in infected cells (Fig. 3*B*). It was shown that sCARfC6.5 as well as sCARf displayed an increased ability to inhibit CAR-dependent Ad infection compared with monomeric sCAR-His_6 protein. The concentrations of sCARfC6.5, sCARf, and sCAR-His_6 needed to block Ad infection by 50% were 3, 6, and 54 nM, respectively. Interestingly, sCARfC6.5 protein displayed Ad infection inhibition efficiency somewhat higher than did sCARf control protein. This experiment validated the utility of trimeric sCAR fusion proteins to block CAR-dependent Ad tropism and, therefore, provided a rationale for additional c-*erb*B-2 targeting studies.

Bispicific sCARfC6.5 Protein Binds to Cellular c-*erb*B-2. Flow cytometry analysis was performed to validate that C6.5 scFv incorporated into recombinant sCARfC6.5 fusion protein retained its ability to bind c-*erb*B-2 oncoprotein at the cell surface. The sCARfC6.5 protein was used to bind to c-*erb*B-2 that was overexpressed on AU-565 breast cancer cells. The MDA-MB-468 breast cancer cell line, previously shown to be c-*erb*B-2-negative, was used as a control. The sCARfC6.5 protein, bound to c-*erb*B-2 displayed at the cell

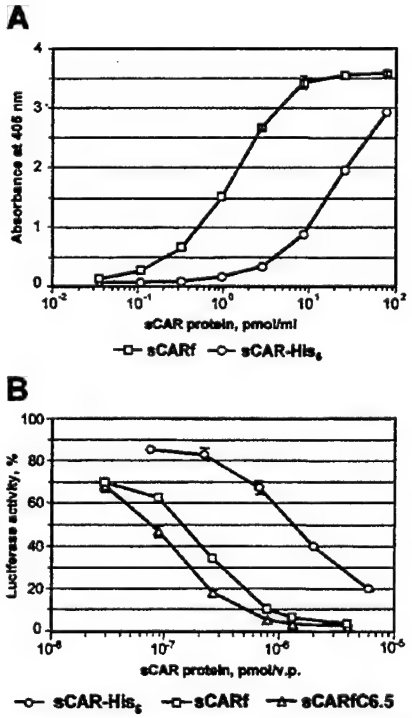

Fig. 3. Analysis of sCAR fusion proteins binding of to Ad fiber knob. *A*, comparison of monomeric and trimeric sCAR protein knob-binding by ELISA. Biotinylated trimeric sCARf and monomeric sCAR-His_6 fusion proteins were incubated at various concentrations with immobilized recombinant Ad5 fiber knob protein. Biotinylated sCAR fusion proteins bound to fiber knob were detected with alkaline phosphatase conjugated with streptavidin. *Each point*, the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols), SDs. *B*, inhibition of Ad infection of CAR-positive cells by sCAR fusion proteins. AdLucGFP vector containing luciferase expression cassette was incubated with either PBS or with increasing amounts of monomeric sCAR-His_6, or trimeric sCARf, or sCARfC6.5 fusion proteins. Viral mixtures were added to monolayers of 293 cells at MOI of 100 v.p./cell. After 30-min incubation to allow virus internalization, the medium was changed and cells were incubated for an additional 20 h at 37°C to allow luciferase expression. Then, cells were lysed, and relative luciferase activity was analyzed. Luciferase activities, detected in cells infected in the presence of sCAR fusion proteins, are shown as *percentages of luciferase activity* registered in control cells infected with AdLucGFP incubated with PBS. Each point represents the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols), SDs.

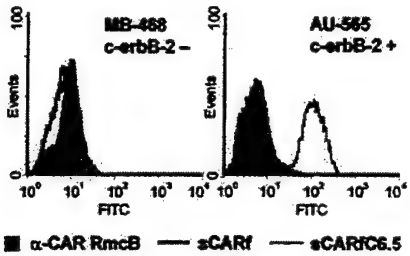

Fig. 4. Confirmation of sCARfC6.5 protein binding to cellular c-*erb*B-2. Trimeric sCARfC6.5 and sCARf fusion proteins were incubated with either c-*erb*B-2-positive AU-565 or c-*erb*B-2-nagative MB-468 cells. The sCAR fusion proteins bound to cells were probed with anti-CAR RmcB MAb and then detected with secondary Alexa 488-labeled goat antimouse antibodies. Binding of sCARfC6.5 protein (*black line*) to c-*erb*B-2-positive AU-565 cells is seen because of the positive staining relative to sCARf control protein (*gray line*) or anti-CAR MAb alone (*spike filled in black*). Representative data from two independent experiments are shown.

surface, presented the CAR ectodomain for antibody detection with primary anti-CAR RmcB MAb (33) followed by a secondary anti-mouse fluorochrome-conjugated antibody. As shown in Fig. 4, incubation of AU-565 cells, naturally low in CAR (Fig. 5), with sCARfC6.5 protein increased cell binding of anti-CAR antibody. In contrast, neither the incubation of AU-565 cells with sCARf control protein nor the incubation of sCARfC6.5 with MDA-MB-468 c-*erb*B-2-negative cells revealed any increase of RmcB MAb antibody binding compared with MAb alone (Fig. 4). Thus, we demonstrated that C6.5 scFv that was incorporated in the context of fusion protein retained its functional ability to recognize cellular c-*erb*B-2 oncoprotein, which enabled sCARfC6.5 protein binding to c-erbB-2-positive cells.

sCARfC6.5 Adapter Protein Mediates c-*erb*B-2-specific Ad Infection. To test the utility of sCARfC6.5 adapter protein for Ad targeting, we evaluated its ability to improve Ad-mediated gene transfer to c-*erb*B-2-positive cells. The established human breast cancer cell lines AU-565, SK-BR-3, BT-474, MCF-7, and MDA-MB-468 and ovarian cancer cell line SK-OV-3 were used to validate the ability of c-*erb*B-2 oncoprotein to mediate Ad infection. Our previous study showed that these cells are relatively refractory to Ad infection.[4] The data were corroborated by flow cytometry analysis that showed either absence or low level of CAR on their cell surface (Fig. 5). Importantly, high levels of c-*erb*B-2 were detected in these cell lines (Fig. 5), which suggested that Ad targeting to c-*erb*B-2 may overcome poor vector susceptibility attributable to the lack of CAR. To determine the optimal adapter protein to virus ratio, sCARfC6.5 protein was titered against a constant dose of AdLucGFP vector (100 v.p./cell) as measured by improvements in gene transfer efficiency. The magnitude of gene transfer augmentation by targeted Ad complexed with sCARfC6.5 adapter was illustrated on selected c-*erb*B-2-positive cell lines and c-*erb*B-2-negative MDA-MB-468 cells compared with untargeted Ad preincubated with sCARf control protein or Ad alone (Fig. 6). Fig. 6*A* shows sCARfC6.5 protein dose-dependent enhancement of gene transfer that was achieved by targeted Ad compared with that achieved by untargeted Ad, as measured by luciferase activity that was detected in infected cells. The sCARfC6.5/Ad ratio providing maximal gene-transfer increase ranged from 1×10^{-7} to 3×10^{-7} pmol/v.p. depending on the cell line tested. As shown in Fig. 6*A*, the sCARfC6.5-targeting protein mediated a 3.4-, 11-, 32-, 47-, and 135-fold enhancement of gene transfer to MCF-7, SK-OV-3, BT-474, SK-BR-3, and AU-565 cells, respectively. The sCARfC6.5 adapter protein provided 1.5- to 17-fold increase of Ad gene transfer compared with Ad alone in most cell lines; however, the levels of improvement in cell transduction were highly variable (Fig. 6*B*). Although c-*erb*B-2-targeted Ad showed a 6-fold enhancement of gene transfer to SK-OV-3 cells and no transduction improvement of MCF-7 cells compared with Ad alone, both of the cell lines demonstrated similar levels of gene transfer and remained relatively Ad refractory. Consistent with the augmentation of the Ad gene transfer to c-*erb*B-2-positive cell lines achieved by sCARfC6.5 targeting protein, untargeted Ad that was complexed with sCARf control protein showed a marked decrease in gene transfer. Importantly, the use of both targeting and control protein to mediate the Ad infection of c-*erb*B-2-negative MDA-MB-468 cells that expressed moderate levels of CAR resulted in an 8-fold decrease of gene transfer. These data strongly indicate that the sCARfC6.5-targeting adapter promoted the Ad infection of CAR-deficient cells specifically via a c-*erb*B-2-dependent pathway.

Augmentation of c-*erb*B-2-targeted Ad Infection Efficiency. The Ad infection of the cells overexpressing c-*erb*B-2 oncoprotein mediated by the sCARfC6.5-targeting protein resulted in the enhancement of luciferase reporter gene expression. This increase in reporter activity could result from an increased number of infected cells or, alternatively, from an elevated level of transgene expression caused by the augmented infection of a limited population of cells. To address this issue, cells were infected with AdLucGFP vector, preincubated with PBS (Ad alone), sCARf (untargeted Ad), or sCARfC6.5 (c-*erb*B-2-targeted Ad) proteins at a sCAR protein:Ad ratio of 2×10^{-7} pmol/v.p. Ad infection efficiency was monitored by direct visualization of GFP expression by fluorescence microscopy. Fig. 7 shows the results of Ad-mediated GFP reporter gene delivery to three representative cell lines: AU-565, SK-BR-3, and BT-474. Infection with AdLucGFP vector alone resulted in a low percentage of GFP-expressing cells, whereas highly increased numbers of infected cells were detected in the case of c-*erb*B-2-targeted Ad. In contrast, infection

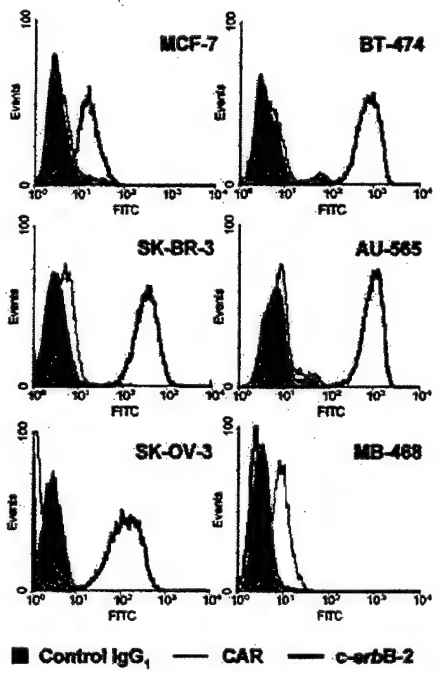

Fig. 5. Expression of CAR and c-*erb*B-2 in cancer cell lines. The breast cancer cell lines MCF-7, BT-474, SK-BR-3, AU-565, and MB-468 and ovarian cancer cell line SK-OV-3 were analyzed for CAR and c-*erb*B-2 expression (by indirect immunofluorescence assay using anti-CAR RmcB and anti-c-*erb*B-2/HER-2/neu Ab-2 MAb, respectively. Positive staining for CAR (*thin black line*) and c-*erb*B-2 (*bold black line*) is seen relative to an isotype control IgG (*spike filled in gray*). Representative data from two independent experiments are shown.

[4] Unpublished observations.

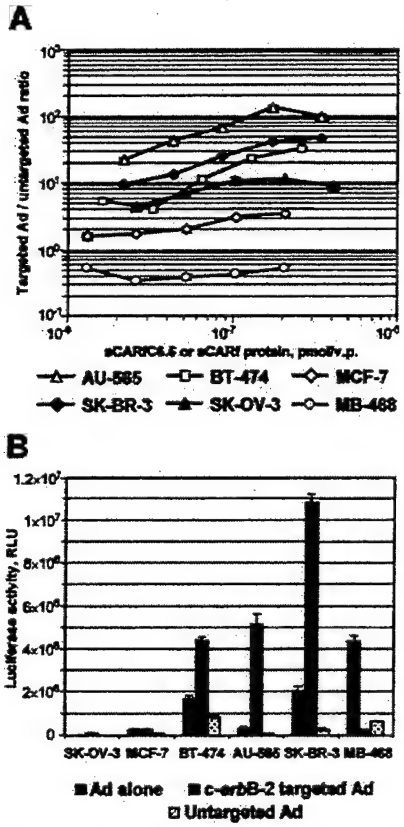

Fig. 6. sCARfC6.5 targeting adapter protein promotes Ad gene transfer to c-erbB-2-positive cells. *A*, determination of optimal sCARfC6.5 adapter protein:Ad ratio. AdLucGFP vector expressing luciferase reporter gene was preincubated with either sCARfC6.5 targeting protein or sCARf control protein at varying concentrations to form c-*erb*B-2-targeted or untargeted viral complexes, respectively. The monolayers of MCF-7, BT-474, SK-BR-3, AU-565, SK-OV-3, and MB-468 cells were infected with targeted or untargeted viral complexes at MOI of 100 v.p./cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as logarithm of ratio of luciferase activities detected in the cells infected with targeted Ad to luciferase activities detected in the cells infected with untargeted Ad complexes formed at the same concentration of each sCAR protein (*Targeted Ad:Untargeted Ad*). Each point represents the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols). SDs. *B*, enhancement of Ad gene transfer by sCARfC6.5 targeting protein. AdLucGFP vector was preincubated with either PBS (*Ad alone*) or one of sCARfC6.5 (*c-erbB-2-targeted Ad*) or sCARf (*untargeted Ad*) proteins at the concentration providing maximal gene transfer augmentation as determined in *A*. The cell monolayers were infected with Ad alone (■), c-*erbB*-2-targeted Ad (■), or untargeted Ad (▨) viral complexes at MOI of 100 v.p./cell and analyzed for luciferase expression 46 h postinfection. Luciferase activities detected in cell lysates are shown as the cumulative mean of triplicate determinations of relative light units (*RLU*) ± SD.

with untargeted Ad showed a decreased infection efficiency compared with that of Ad alone, which resulted in few or no fluorescent cells. Infection of c-*erb*B-2-negative MDA-MB-468 cells with targeted Ad did not show any improvement of infection efficiency compared with that achieved by untargeted Ad or by Ad alone (data not shown). These results were consistent with data obtained for Ad-mediated luciferase gene delivery (Fig. 6).

DISCUSSION

One of the major challenges facing Ad gene delivery systems is the modification of viral native tropism to confer targeting capability on selected cell types. The limitation of Ad vectors associated with broad CAR-dependent tropism and inefficient infection of CAR-deficient cells could be solved by Ad targeting to a novel receptor overexpressed in the cells of interest. In this study, we explored the utility of a recombinant adapter protein to achieve modification of Ad tropism. The use of adapter molecules to mediate cell-specific Ad infection relies on the following: (*a*) the ability to be produced and purified at preparative amounts and retain stable structure; (*b*) the ability to provide efficient linkage to the v.p. while blocking Ad native tropism; and (*c*) the ability to achieve binding to cell-specific receptors, thus generating a novel tropism. We previously developed a targeting approach based on the use of the sCAR ectodomain fused with EGF, which allowed both the blocking of CAR-dependent tropism and the Ad targeting to the cells overexpressing EGFR (29). To extend this approach, we engineered an adapter protein, sCARfC6.5, comprised of sCAR fused with a trimerization domain and a scFv against c-*erb*B-2 oncoprotein to target cancer cell types, and a sCARf control protein that lacked the anti-c-*erb*B-2 scFv targeting moiety. A trimerization domain derived from phage T4 fibritin protein was incorporated into the design of the sCAR fusion proteins to achieve tight viral linkage by virtue of trivalent binding to the Ad fiber knob. Both the sCARfC6.5 targeting protein and the control sCARf protein were expressed in insect cells and purified at preparative amounts by affinity chromatography. An analysis of purified fusion proteins showed that sCARf and sCARfC6.5 retain their designed composition and maintain stable trimeric structure. As expected, trimerization of sCAR proteins resulted in the augmentation of knob-binding efficiency by at least 20-fold compared with that achieved by the monomeric sCAR-His_6 protein. This result is consistent with a kinetic analysis showing that, in contrast to the high on/off interaction rates between CAR D1 domain and Ad2 fiber knob, the binding of the knob domain to three D1 molecules simultaneously leads to a low overall off rate and K_d of ~1 nM (16). We then evaluated whether trimerization would result in an improved ability of sCAR proteins to block Ad infection. An infection inhibition assay demonstrated that the concentrations (3 and 6 nM, respectively) of trimeric sCARfC6.5 and sCARf proteins that were needed to inhibit Ad-mediated gene transfer by 50% were, respectively, 18- and 9-fold lower than the concentration (54 nM) of monomeric sCAR-His_6 protein that was needed. These data proved the utility of trimeric sCAR adapter proteins in blocking CAR-dependent Ad infection. The fact that T4 fibritin forms homotrimers that are resistant to dissociation by SDS and digestion by trypsin (37, 38) suggests that the incorporation of a fibritin polypeptide in the context of sCAR fusion might provide a highly stable trimeric structure compatible with *in vitro*, and likely *in vivo*, Ad targeting schemes. We showed that, in addition to its ability to block Ad infection, the sCARfC6.5 adapter protein binds to cellular c-*erb*B-2 oncoprotein and, therefore, enables Ad targeting via a CAR-independent pathway. In ovarian and breast cancer cell lines overexpressing c-*erb*B-2, the ability of the c-*erb*B-2 oncoprotein to mediate Ad infection was illustrated by markedly increased levels of gene transfer and numbers of infected cells. Thus, the use of the sCARfC6.5 adapter protein overcomes the barrier of CAR deficiency by retargeting the Ad infection via the c-*erb*B-2 oncoprotein and provides a ≤2-fold enhancement of gene transfer efficiency in comparison with that provided by the sCARf control protein. Our observation that the use of the sCARfC6.5 adapter decreased the Ad infection efficiency in c-*erb*B-2-negative MDA-MB-468 cells that expressed moderate levels of CAR suggests the high specificity of this Ad-targeting approach. Importantly, the presence of three scFvs in each trimeric sCARfC6.5 molecule likely contributes to the increase in apparent receptor affinity attributable to polyvalent binding and contributes, therefore, to the efficiency of Ad targeting. In fact, the presence of additional binding sites prolongs the association of scFv-based molecules with tumor cells *in vitro* and *in vivo*. Studies of bivalent diabody molecules that were constructed from C6.5 scFv demonstrated a more highly prolonged association (60-fold) with c-*erb*B-2

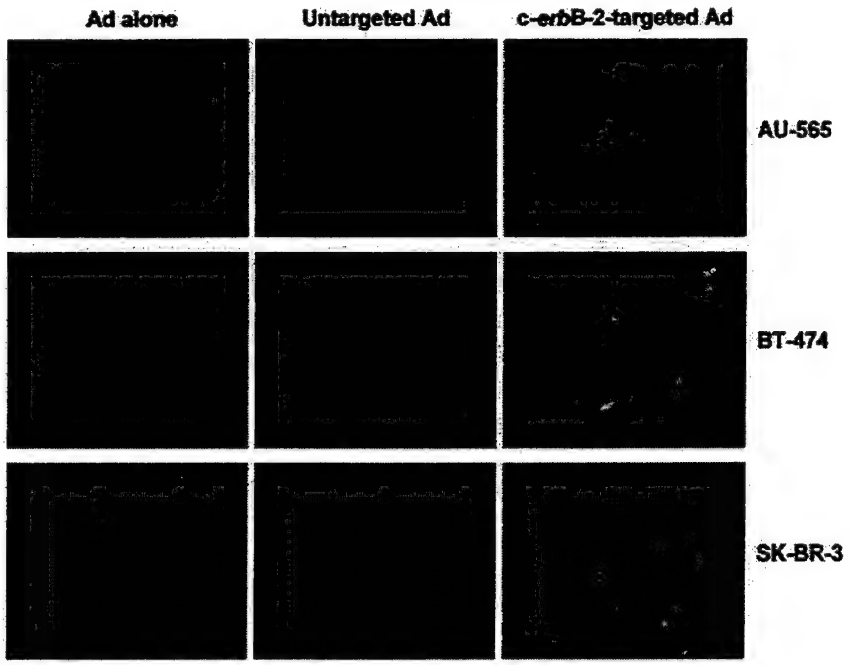

Fig. 7. The use of sCARfC6.5 targeting protein improves efficiency of Ad infection of c-erbB-2-positive cells. AdLucGFP vector expressing GFP was preincubated with PBS (*Ad alone*), sCARfC6.5 adapter protein, or sCARf control protein at the concentration of 2×10^{-7} pmol/v.p to form c-*erb*B-2-targeted or untargeted viral complexes, respectively. The monolayers of SK-BR-3, BT-474, and AU-565 cells were infected with Ad alone, untargeted Ad, or c-*erb*B-2-targeted Ad complexes at MOI of 100 v.p./cell. Infected cells expressing GFP were detected 24 h postinfection by fluorescence microscopy.

on the surface of SK-OV-3 cells and more tumor-retained diabodies (6.5-fold) when compared with scFv monomer (41).

The use of trimeric sCAR-ligand fusion proteins or recombinant adapter molecules sharing a trimeric design to confer Ad targeting to specific cell types may augment the utility of current Ad vectors. In addition, the availability of scFvs with defined specificities to tumor antigens offers the flexibility of ligand substitution to expand the targeting capabilities of Ad vectors. Of note, Ad targeting by means of bispecific antibody conjugates was shown to achieve direct therapeutic goals in *in vivo* models relevant to human clinical cancer gene therapy schemes (42–45). Thus, modification of Ad tropism based on the use of recombinant adapters could facilitate target-directed infection, which will reduce the effective therapeutic viral dose, thereby decreasing the immediate toxicity and increasing the safety and efficiency of Ad vectors.

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Advances in Brief

Targeting Oncolytic Adenoviral Agents to the Epidermal Growth Factor Pathway with a Secretory Fusion Molecule[1]

Akseli Hemminki,[2] Igor Dmitriev, Bin Liu, Renee A. Desmond, Ramon Alemany, and David T. Curiel

Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery, and the Gene Therapy Center [A. H., I. D., B. L., R. A., D. T. C.], and Comprehensive Cancer Center Biostatistics Unit [R. A. D.], University of Alabama at Birmingham, Birmingham, Alabama 35294-3300

Abstract

Cancer gene therapy with conditionally replicating adenoviruses is a powerful way of overcoming low tumor transduction. However, one of the main remaining obstacles is the highly variable level of the coxsackie-adenovirus receptor expression on human primary cancers. In contrast, the epidermal growth factor receptor (EGFR) is overexpressed in various tumor types, and its expression correlates with metastatic behavior and poor prognosis. We constructed an adenovirus expressing a secretory adaptor capable of retargeting adenovirus to EGFR, resulting in a more than 150-fold increase in gene transfer. A replication-competent dual-virus system secreting the adaptor displayed increased oncolytic potency *in vitro* and therapeutic gain *in vivo*. This approach could translate into increased efficacy and specificity in the treatment of EGFR overexpressing human cancers.

Introduction

CRADs[3] are a promising and novel way of overcoming low tumor transduction, which is the main obstacle preventing effective gene transfer and therapeutic effect in clinical applications of cancer gene therapy (1). However, one of the main reasons why the unparalleled transduction efficacy of Ads has not translated into similar results in humans is the variable level of the CAR on primary cancers (2–9) *in vivo*. CAR is ubiquitously expressed on normal epithelial tissues and is the main receptor mediating binding of the most commonly used Ad serotypes 2 and 5. Expression of CAR may be the major factor determining the rate of transduction (4, 6, 9–11). Importantly, recent evidence (11) suggests that CAR expression may inversely correlate with the malignant potential of tumors, resulting in low infectivity of highly aggressive tumors. In contrast to the expression profile of CAR, EGFR, the prototype of cancer-associated receptors, is commonly overexpressed in many if not most carcinomas with correlation to metastatic behavior and poor prognosis (12). A powerful approach for increasing tumor transduction could be combining the tissue-penetrating capability of CRADs with the transductional control provided by retargeting moieties. In support of this hypothesis, an artificial receptor system has been used to demonstrate that the effect of Ad dispersion and subsequent oncolysis critically depends on receptor expression (13). We have constructed a novel virus that mediates secretion of a fusion molecule consisting of the extracellular domain of CAR and EGF. We then explored the capability of the sCAR-EGF to retarget Ad to EGFR. Finally, we demonstrated that infection of cancer cells with a sCAR-EGF-retargeted replication-competent dual-virus system resulted in increased oncolysis *in vitro* and a therapeutic benefit *in vivo*.

Materials and Methods

Viruses. For construction of AdsCAR-EGF, a replication-deficient Ad with sCAR-EGF in E1, the gene coding for sCAR-EGF was cloned from pFBshCAR-EGF (14) into pShuttle-CMV (Quantum, Montreal, Quebec, Canada). Homologous recombination with pAdEasy-1 (Quantum) was performed in *Escherichia coli*, followed by confirmation of structure with *Eco*RV and *Pac*I digestions, PCR, and sequencing of the transgene (data not shown). The viral genome was transfected into 293 cells for plaque purification, followed by cesium chloride purification and standard titering with OD260 and plaque assay. Resulting titers were 3.8×10^{11} VPs/ml and 1.0×10^{10} plaque-forming units/ml, ratio = 38.4 VPs/plaque-forming unit. Large-scale preparations of AdCMVLuc (a nonreplicating Ad-expressing luciferase; courtesy of Dr. Robert Gerard, Texas Southwestern Medical Center, Dallas, TX), AdsCAR6H (a nonreplicating Ad-mediating secretion of sCAR6H),[4] and Δ24 (an Ad with a 24-bp deletion in *E1A*, allowing selective replication in cells mutant in the *Rb-p16* pathway; Ref. 15) were performed with standard methods on 293 cells (or A549 cells for Δ24).

CRAdsCAR-EGF denotes a replication-competent, sCAR-EGF-secreting dual-virus system consisting of equal VPs of AdsCAR-EGF and Δ24 mixed immediately before administration to cells. CRAdCMVLuc is the respective combination of Δ24 and AdCMVLuc. Δ24 has been characterized previously (15). Validating the dual-virus strategy, it has been demonstrated that transcomplementation of E1 proteins from a plasmid or replication-competent virus results in replication of E1-deleted viruses present in the same cell (9, 16–18).

Cell Lines. 293 cells were purchased from Microbix (Toronto, Ontario, Canada). A549 (lung cancer), HeLa (cervical cancer), U118 (glioma), A431 (squamous cell skin cancer), BT474, and MB-453 (breast cancer) were obtained from the American Type Culture Collection (Rockville, MD), and SKOV3.ip1 cells (ovarian cancer) are from Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX). Cell lines were propagated in the recommended conditions.

Protein Detection. HeLa cells were infected overnight with 50 VP/cell, and BT474 and MB453 cells were infected with 500 VP/cell of AdsCAR-EGF, AdsCAR6H, and AdCMVLuc. Supernatants were collected at 48 h, and cellular debris was removed by centrifugation. Dilutions in a volume of 300 μl were transferred onto a nitrocellulose membrane using the Bio-Dot apparatus (Bio-Rad). BSA (3%) was used for blocking, followed by detection with a 1:5000 polyclonal mouse anti-CAR antibody (14) and 1:2000 goat antimouse alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in 3% BSA. Western blot analysis on the supernatants was performed with standard methodology in a 12% two-phase gel, and proteins were detected as above. Baculovirus-expressed and -purified sCAR-EGF and sCAR6H (14) were used as controls.

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[2] To whom requests for reprints should addressed, at Division of Human Gene Therapy, Gene Therapy Center, WTI #602, 1824 6th Avenue S., University of Alabama at Birmingham, Birmingham, AL 35294-3300. Phone: (205) 975-2896; Fax: (205) 975-8565; E-mail: akseli@uab.edu.

[3] The abbreviations used are: CRAD, conditionally replicative adenovirus; Ad, adenovirus; CAR, coxsackie-Ad receptor; EGF, epidermal growth factor; EGFR, EGF receptor; sCAR-EGF, secretory CAR-EGF fusion molecule; CMV, cytomegalovirus immediate early promoter; VP, viral particle; sCAR6H, secretory CAR with 6-histidine tag.

[4] I. Dmitriev, unpublished observations.

Retargeting Assays. SKOV3.ip1, U118, and A431 cells were infected in duplicate with AdCMVLuc preincubated for 30 min with aliquots of supernatants (see above). Twenty VP/cell were used for SKOV3.ip1, whereas 200 VP/cell were used for U118 and A431. Luciferase assay was performed 48 h later (Luciferase Assay System; Promega, Madison, WI).

Cell-killing Assays. SKOV3.ip1 cells were plated in triplicate and infected with Δ24 or AdCMVLuc for 1 h. Aliquots (25 μl) of AdsCAR-EGF or AdCMVLuc supernatant (from protein detection assays) were added each day. At 20 days, cells were fixed with formalin and stained with crystal violet.

Next, A431 and SKOV3.ip1 cells were infected with the CRAdCMVLuc or CRAdsCAR-EGF for 1.5 h. After incubating for 20 days with medium changes, the remaining cells were fixed and stained as above.

Preliminary toxicity analysis was performed by infecting A431 and SKOV3.ip1 cells with 50, 100, 200, 500, and 1000 VP/cell of AdsCAR-EGF and AdsCAR6H, followed by medium changes every 2 days for 20 days.

Animal Experiments. Initially, A431 cells were infected *ex vivo* with 50 VP/cell of CRAdsCAR-EGF for 1 h followed by a 5-h incubation. Cells were then collected and mixed with uninfected cells, and a total of 10^7 cells were injected into flanks of athymic CD-1/nu mice (Charles River Laboratories, Wilmington, MA; n = five/group). Tumor size was determined as the mean of the shortest and longest diameter (to avoid variability attributable to difficulty with estimation of height).

To compare CRAdsCAR-EGF to CRAdCMVLuc *in vivo*, s.c. tumors were established by injecting 5×10^6 A431 cell into both flanks of athymic mice (n = five/group). When tumors were ~5 mm ("day 0"), viruses were injected intratumorally in a 15-μl volume and tumors were measured as above. Each mouse was checked daily for the absence of pain or distress (19).

Statistics. Upon termination of the experiment, mean tumor size and SDs were calculated for each group of animals for each time point. The nonparametric change-point test (20) was used to show a systematic change in the pattern of observations as opposed to fluctuation attributable to chance. The mixed model (21) was used to longitudinally model the data on each tumor. The variance covariance structure was determined by using Akaike's Information Criteria (22). The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth.

Results

Infection of Cells with AdsCAR-EGF Results in Secretion of sCAR-EGF. Initially, infection of high EGFR HeLa cells (23) with AdsCAR-EGF produced no evidence of secretion, whereas sCAR6H was secreted (Fig. 1). With low EGFR-expressing cells MB453 and BT474 (14, 24), secretion of sCAR-EGF was detected. The amount of protein was estimated at 110 ng/ml (75-cm^2 flask; 12 ml of medium). Western blot confirmed secretion (Fig. 1*B*). The altered migration rate of the protein in comparison with baculovirus-expressed sCAR-EGF perhaps resulted from altered charge caused by different glycosylation by insect cells. A preliminary investigation on sCAR-EGF toxicity was performed by infecting SKOV3.ip1 and A431 cells with various amounts of AdsCAR-EGF and AdsCAR6H without significant differences in cell viability (data not shown).

Secreted sCAR-EGF Mediates Retargeting of Ad to EGFR. Aliquots of supernatant from AdsCAR-EGF-infected BT474 cells were incubated with AdCMVLuc. The virus-supernatant mix was used for infection of SKOV3.ip1, U118, and A431 cells, which display moderate (SKOV3.ip1 and U118) to high (A431) EGFR expression and moderate (A431) to low (SKOV3.ip1 and U118) CAR expression (4, 14). A supernatant dose-dependent increase in luciferase expression was seen, with the highest readings 17.1-, 20.2-, and 158-fold higher than without retargeting for SKOV3.ip1, U118, and A431 cells, respectively (curves with triangles in Fig. 2).

With the highest amounts of supernatant from AdsCAR6H-infected cells, luciferase expression was reduced to 73%, 48%, and 65% (on SKOV3.ip1, U118, and A431, respectively) of the highest values for the series (curves with squares in Fig. 2). sCAR6H binds to Ad fiber but does not mediate binding to EGFR, thus modeling blockage of CAR-binding with sCAR-EGF.

Retargeting Replication-competent Ad to EGFR Results in Increased Cell Killing *in Vitro*. To validate Δ24 replication in SKOV3.ip1 cells, infections were performed at 0, 0.01, 0.1, or 1 VP/cell, and aliquots of supernatant (from BT474 cells infected with

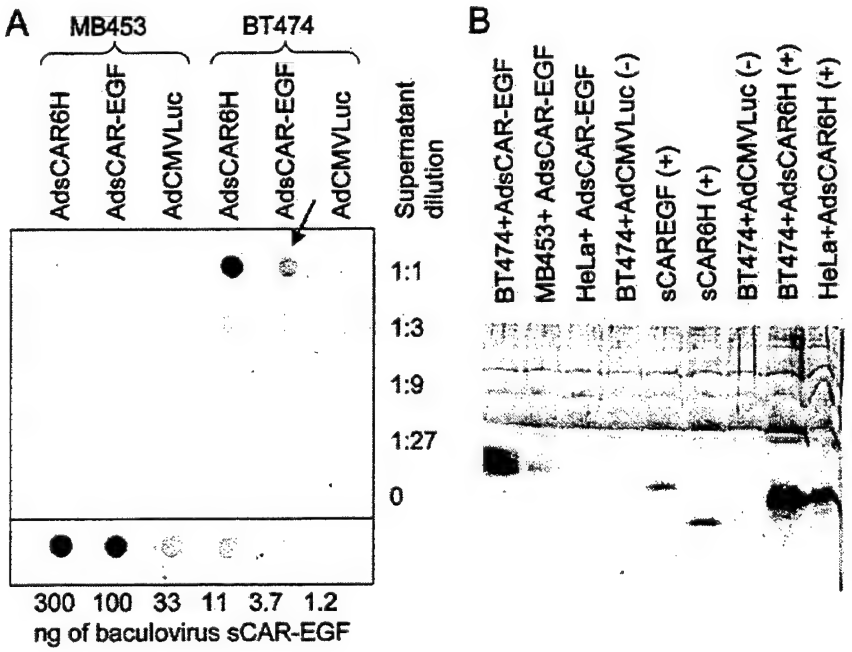

Fig. 1. Secretion of sCAR-EGF from human cancer cells infected with AdsCAR-EGF. *A*, supernatant from MB453 and BT474 (both low EGFR breast cancer lines) cells infected with AdsCAR6H (codes for human CAR ectodomain, positive control), AdsCAR-EGF, or AdCMVLuc (negative control) was centrifuged and then transferred onto a membrane. *Arrow*, the signal for sCAR-EGF. When compared with known amounts of sCAR-EGF (*lowest row*), the amount of secretion could be estimated at 110 ng/ml. *B*, Western blot suggested that the sCAR-EGF secreted from BT474 and MB453 cells (*Lanes 1–2*) was close in size to baculovirus-expressed sCAR-EGF (*Lane 5*). High EGFR HeLa cells (*Lane 3*) did not show evidence of sCAR-EGF secretion, but the positive control sCAR6H was secreted (*Lane 9*). −, supernatants collected from cells infected with AdCMVLuc (*Lanes 4 and 7*). These serve as negative controls. +, the positive controls, including *Lane 8*, which has supernatant from BT474 cells infected with AdsCAR6H. sCAR-EGF and sCAR6H (*Lanes 5–6*) are purified baculovirus-expressed proteins.

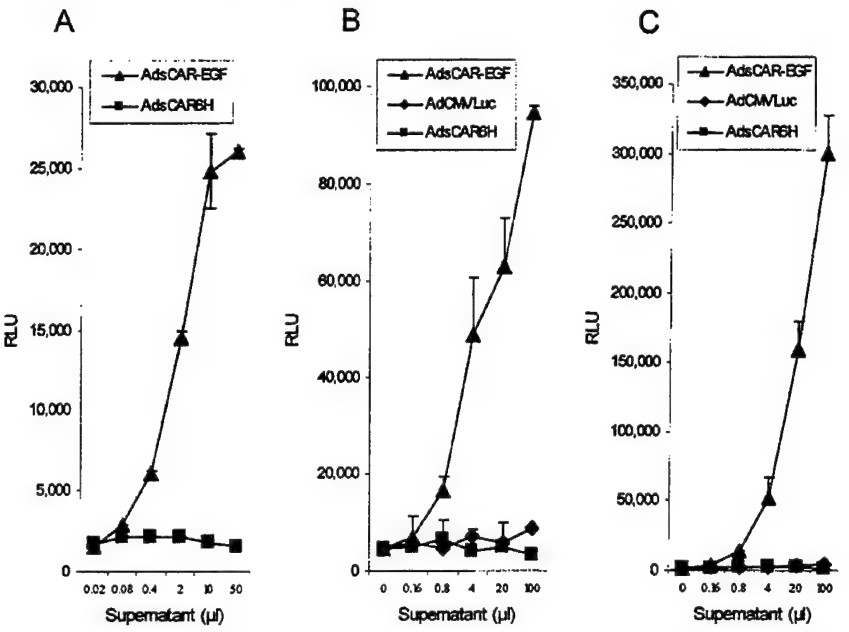

Fig. 2. Secreted sCAR-EGF mediates retargeting of Ad to EGFR. Increasing amounts of supernatant collected from cells infected with AdsCAR-EGF (curves with ▲) were incubated with AdCMVLuc, and then the mix was added to (*A*) SKOV3.ip1 (ovarian cancer), (*B*) U118 (glioma), or (*C*) A431 (squamous cell skin cancer) cells. These cells express moderate to high EGFR and thus resemble many aggressive human cancers. Supernatant from cells infected with AdCMVLuc (◆) or AdsCAR6H (■) were used as controls. Relative light units (*RLU*) are means of duplicate experiments (±1 SD). With the highest amounts of supernatant, luciferase readings were 17.1-fold (*A*), 20.2-fold (*B*), and 158-fold (*C*) higher with retargeting. The slope of the AdsCAR-EGF curves suggests that maximum retargeting potential was not reached.

AdsCAR-EGF or AdCMVLuc) were added daily. At 20 days, cell killing and partial loss of monolayer was seen only with cells that had been infected with 1 VP/cell and subjected to AdsCAR-EGF/BT474 supernatant (data not shown).

To study the effect of continuous sCAR-EGF secretion on the oncolytic potential of CRADs, we infected SKOV3.ip1 and A431 cells with the CRAdsCAR-EGF and CRAdCMVLuc dual-virus systems. On both cell lines, infection with CRAdsCAR-EGF resulted in cell killing with one to two orders of magnitude less virus than with CRAdCMVLuc (Fig. 3).

Targeting Replicative Ad to EGFR Results in a Therapeutic Advantage *in Vivo*. Various proportions of infected and uninfected A431 cells were mixed and injected s.c. (Fig. 4*A*). One percent of infected cells was sufficient to inhibit tumor growth, and 5% or more resulted in healing of tumors. None of the mice showed signs of illness or distress, suggesting that the secretion of sCAR-EGF did not cause overt toxicity.

To evaluate sCAR-EGF retargeting *in vivo*, CRAdsCAR-EGF or CRAdCMVLuc were administered with a single intratumoral injection into established A431 tumors (Fig. 4, *B–C*). The change-point test (20) revealed that the tumor growth pattern changed at 13 days for 10^9 VP CRAdsCAR-EGF ($P = 0.0045$), 21 days for 10^9 VP CRAdCMVLuc ($P = 0.0012$), 17 days for 10^8 VP CRAdsCAR-EGF ($P = 0.0026$), and 25 days for 10^8 VP CRAdCMVLuc ($P = 0.0011$), *i.e.*, 8 days earlier for CRAdsCAR-EGF with both doses.

The change-point test and the test of fixed effects (22) showed that there was a significant correlation between observations of tumor size and time ($P < 0.0001$ for all of the groups). A polynomial equation was fit for each group, thereby creating a mathematical model for each growth pattern ("modeled" in Fig. 4). The mixed model (22) was used

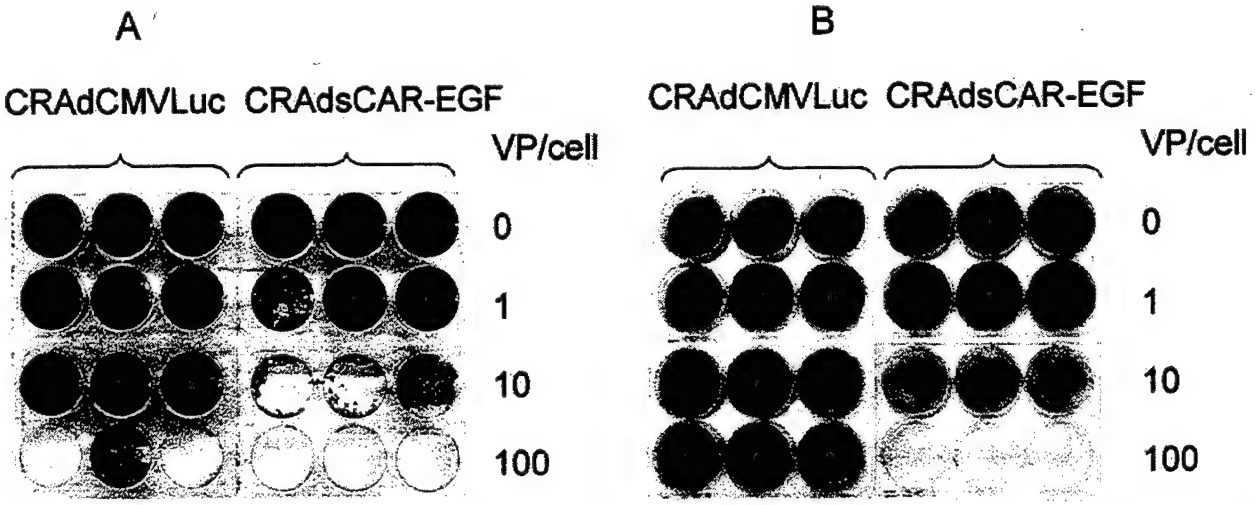

Fig. 3. Targeting oncolytic Ad to EGFR with sCAR-EGF results in an increased oncolytic effect. (*A*) SKOV3.ip1 or (*B*) A431 cells were infected with CRAdsCAR-EGF, a replication-competent sCAR-EGF-secreting dual-virus system. Oncolytic potency was compared with CRAdCMVLuc, which is isogenic in regard to replicativity but does not secrete a retargeting molecule. A similar effect on cells was observed with 1 VP/cell of CRAdsCAR-EGF as with 100 VP/cell of CRAdCMVLuc, suggesting increased oncolysis because of sCAR-EGF secretion-mediated EGFR targeting.

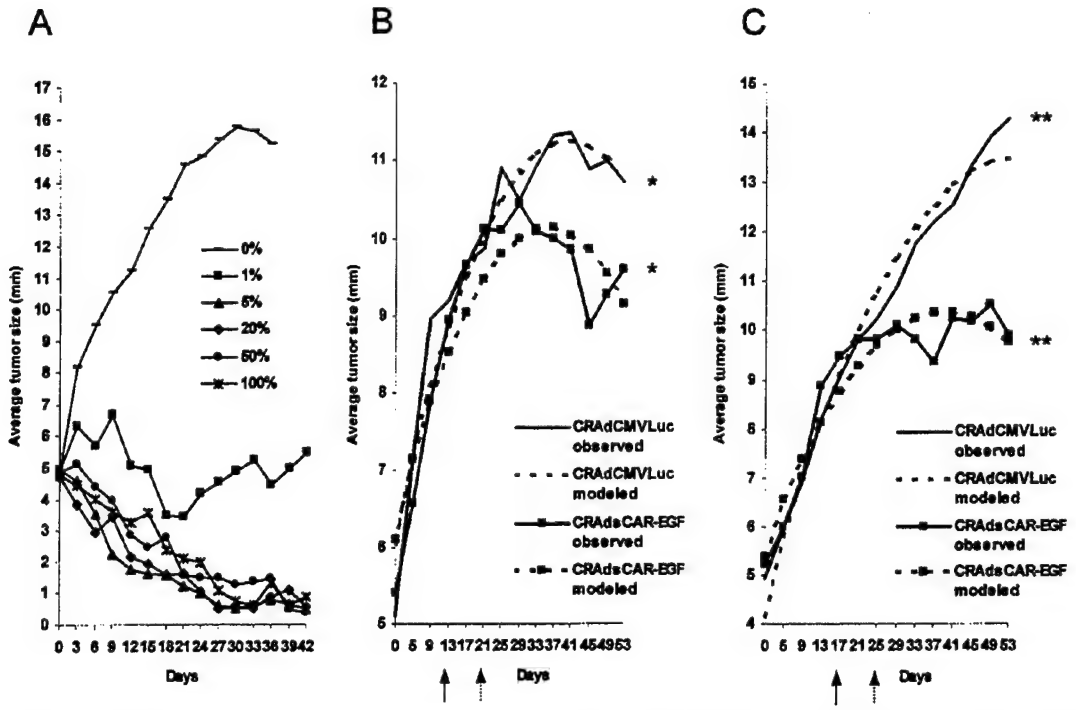

Fig. 4. sCAR-EGF secretion results in therapeutic efficacy *in vivo*. *A*, various percentages of A431 cells were infected *ex vivo* with CRAdsCAR-EGF and mixed with uninfected cells. One percent of infected cells was sufficient to inhibit tumor growth, whereas 5% or more resulted in healing of mice. Single intratumoral injections of (*B*) 10^9 or (*C*) 10^8 VP of CRAdsCAR-EGF or CRAdCMVLuc dual-virus systems were performed into established A431 xenografts. *Arrows* (*solid* for CRAdsCAR-EGF, *dotted* for CRAdCMVLuc) indicate the change-point in tumor growth characteristics, which represents the time point when oncolysis changes the initial pattern of tumor growth. On the basis of tumor size measurements (*observed*), the growth patterns were mathematically modeled for statistical comparison (*modeled*), and CRAdsCAR-EGF was found to be more oncolytic. *, $P < 0.05$; **, $P < 0.0001$.

to allow for the curvature of the plots, which was caused by the opposite effects of oncolysis and cell division. For the mice that received injections with 10^9 VP, groups were significantly different from day 29 onwards ($P < 0.05$; Fig. 4*B*). When 10^8 VP was used (Fig. 4*C*), the differences were significant from day 25 onwards ($P = 0.0066$ and 0.0003 on days 25 and 29, respectively, and < 0.0001 on days 33–53).

Discussion

In this study, we report construction of the first human Ad secreting a paracrine adaptor molecule. Secretion of sCAR-EGF was demonstrated with low EGFR cells (*Lanes 1 and 2*, Fig. 1), but not with high EGFR HeLa cells (23). In contrast, secretion was detected when HeLa cells were infected with AdsCAR6H, which codes for ectodomain of CAR but not EGF (*Lane 9*, Fig. 1*B*). EGF exhibits high affinity binding to EGFR, which leads to rapid internalization but no recycling of the receptor-ligand complex (12). Thus, perhaps secreted sCAR-EGF also internalizes. Alternatively, binding without internalization would also limit the amount of sCAR-EGF in the supernatant.

Supporting the capacity of sCAR-EGF to mediate binding and subsequent internalization of Ad, supernatant containing the fusion molecule resulted in dose-dependent increases in marker gene expression (Fig. 2). The shape of the curves suggests that the upper limit of retargeting potential was not reached. In an *in vitro* system, it is difficult to assess the capability of a secreted fusion molecule to block fiber-CAR interaction, because in the absence of CAR binding, uptake of Ad into cells can also occur via alternative mechanisms (10). However, we observed up to 52% reduction in luciferase expression with sCAR6H, which could translate into partial blocking of CAR-mediated internalization (into normal cells) by sCAR-EGF *in vivo*, but additional studies are needed.

We used a dual-virus system (CRAdsCAR-EGF) to evaluate the combination of oncolysis and EGFR targeting and saw dramatically increased killing of cells relatively low in CAR but high in EGFR expression, a combination commonly seen with primary cancer cells (Fig. 3). *In vitro*, the isogenic control virus (CRAdCMVLuc) is expected to enter cells even if they are low in CAR (10). The observed difference in oncolysis may result from more rapid internalization of the retargeted virus because of a higher number of receptors. In a living organism, extracellular viruses are at risk for neutralization by immune defenses or being swept away into organs responsible for Ad clearance. Thus, the advantage of rapid binding and internalization could be more pronounced.

s.c. xenografts are a stringent model for testing an oncolytic effect, because viral replication is balanced against rapid tumor growth. Here, we demonstrated significantly improved therapeutic efficacy of CRAdsCAR-EGF in comparison with the isogenic control not secreting sCAR-EGF (Fig. 4).

No signs of sCAR-EGF-causing toxicity were evident when cells were infected with AdsCAR-EGF in comparison with AdsCAR6H. When sCAR-EGF was added daily to SKOV3.ip1 cells infected with a CRAD, no evidence of toxicity to cells was seen. Moreover, obvious signs of toxicity were absent in mice whose xenografts were infected with CRAdsCAR-EGF. Additional studies will show whether the adaptor molecule has an effect of cell growth or whether there is toxicity *in vivo*. Also, it remains to be seen whether sCAR-EGF mediates Ad internalization via the EGFR pathway or merely substitutes for CAR in binding Ad for the native entry mechanism via penton base arginine-glycine-aspartic acid and cellular integrins.

This is the first report of a retargeting molecule secretory from human cells, but this strategy could be feasible with various high-affinity, cancer-specific ligands. Because rapid screening methods allow recognition of large numbers of cancer-specific features, unlimited possibilities for retargeting with secretory-targeting moieties

may soon be available. The dual-virus system used here provides a useful model for rendering AdsCAR-EGF replicative and investigating the combination of oncolysis and retargeting, but efficacy could be improved when *sCAR-EGF* is genetically incorporated into a CRAD.

In conclusion, we show that retargeting of replicating Ad to a receptor overexpressed in cancers is a powerful way of increasing tumor transduction and allows overcoming the lack of the primary Ad receptor. Clinical translation of this approach may be effective in treatment of a variety of human cancers that overexpress EGFR.

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Gene Therapy (2002) 9, 1189–1193

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BRIEF COMMUNICATION

Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells

AV Pereboev[1,2], CK Asiedu[4], Y Kawakami[1,2], SS Dong[4], JL Blackwell[1,2], EA Kashentseva[1,2], PL Triozzi[2,3], WA Aldrich[3], DT Curiel[1,2], JM Thomas[2,4] and IP Dmitriev[1,2]

[1]*Division of Human Gene Therapy, Departments of Medicine, Pathology and Surgery, University of Alabama at Birmingham, Birmingham, AL, USA;* [2]*Gene Therapy Center, University of Alabama at Birmingham, Birmingham, AL, USA;* [3]*Division of Hematology-Oncology, University of Alabama at Birmingham, Birmingham, AL, USA; and* [4]*Division of Transplantation, University of Alabama at Birmingham, Birmingham, AL, USA*

A promising approach to immunotherapy involves the loading of dendritic cells (DCs) with genetic material to facilitate sustained expression of a relevant antigen in this population of potent antigen presenting cells (APC). Viral vectors such as adenovirus (Ad) have been used for this purpose. Existing methods for DC infection are limited by lack of specificity and a requirement for DC exposure to high viral doses. Targeting of Ad to DCs with bispecific antibodies has significantly augmented levels of transgene expression. Genetic fusion of the extracellular portion of coxsackievirus-adenovirus receptor (CAR) to cell-specific ligands has also proved successful in targeting Ad to cells of interest. We report here the production and primary characterization of a new fusion protein comprising the ecto-domain of CAR connected to a single chain antibody (scFv) G28-5 against human CD40 present on the surface of DCs. We demonstrate that the fusion protein (CAR/G28) specifically interacts with both recombinant Ad fiber knob and the ecto-domain of human CD40 in a binding assay (ELISA). Finally, we show that the CAR/G28 fusion protein promotes highly efficient transduction of DCs of both rhesus monkey and human origin.

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Keywords: *CAR; CD40; scFv; dendritic cells; adenovirus; transduction enhancement*

Dendritic cells play a central role in controlling immunity. Antigen is initially processed by immature DCs, which subsequently undergo maturation involving phenotypic and functional changes that enable DC to mediate specific T and B cell activation in a highly effective manner. In tumor-bearing hosts, antitumor immunity can be compromised because tumor antigens fail to elicit immune mechanisms to resist tumor growth. This outcome is possibly related to a deficiency of DC expressing tumor antigens. Accordingly, strategies to vaccinate against tumor-specific antigens utilizing DC are being developed to bolster anti-tumor immunity.[1]

Loading of DC with antigens can be achieved by incubation with antigen-specific peptides[2,3] or cell lysates.[3] Alternatively, antigen expression in DC can be induced by gene transfer using either RNA[4] or DNA with nonviral[1,5] or viral[1,6] vectors. Among the viral vectors, Ad is notable for efficient in vitro and *in vivo* gene transfer and expression, which is independent of target cell replication. However, the relative resistance of DCs to Ad infection due to lack of CAR expression on DC surface[7] is an ostensible shortcoming for this approach. Efficient DCs transduction with Ad has been reported, but high viral doses and prolonged exposure of the DCs to virus were required.[8] Ad transduction can also be facilitated with liposomes,[9] but toxicity for DCs is a potential problem. Thus, there is a need to improve the specificity of targeting Ad vectors to DC in the gene therapy field.

A recently reported approach to achieve directed Ad-based gene transfer to DCs utilized a chimeric Ad of serotype 5 in which the fiber knob region was replaced with that of Ad of serotype 35.[10] However, optimal transduction efficiency with such chimeric virus required a high virus-to-cell ratio (multiplicity of infection, MOI). An alternative approach of Ad targeting to DCs was developed in our laboratory using a chemical conjugate comprising a F(Ab) fragment of a monoclonal antibody raised against Ad serotype 5 fiber and a whole mAb against the DC marker protein CD40.[7] This bispecific antibody demonstrated high transduction efficiency of DCs at much lower viral doses. Notably, this DC-targeted, Ad infection strategy also lead to phenotypic changes reflecting DC maturation.

Recombinant proteins offer a number of technological advantages including simplified production and purification when compared with chemical conjugates. In addition, during chemical conjugation only a fraction of the input component will yield a functionally active

Correspondence: AV Pereboev, Gene Therapy Center, University of Alabama at Birmingham, 406 BMR-2 901 19th Street South, Birmingham Alabama 35294, USA
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RESEARCH ARTICLE

Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors

JG Wesseling[1], PJ Bosma[1], V Krasnykh[3], EA Kashentseva[3], JL Blackwell[3], PN Reynolds[3], H Li[3], M Parameshwar[3], SM Vickers[4], EM Jaffee[5], K Huibregtse[2], DT Curiel[3] and I Dmitriev[3]

[1]*Department of Experimental Hepatology,* [2]*Department of Gastroenterology and Liver Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands;* [3]*Division of Human Gene Therapy, Departments of Medicine, Pathology, and Surgery and the Gene Therapy Center and* [4]*Department of General Surgery, School of Medicine, University of Alabama at Birmingham, Birmingham, AL; and* [5]*Department Of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA*

In this study we analyzed two ways of retargeting of Ad-vectors to human pancreatic carcinoma with the aim of enhancing the gene transfer efficiency. First, we analyzed the expression of the epidermal growth factor receptor (EGFR) on primary, as well as established pancreatic carcinoma cells by flow cytometry which revealed high expression levels of EGFR on the surface of these cells. We showed that EGFR-retargeted entry pathway using a bispecific fusion protein formed by a recombinant soluble form of truncated Coxsackie and Adenovirus Receptor (sCAR) genetically fused with human EGF (sCAR-EGF) redirects them to the EGFR leading to an enhanced gene transfer efficiency to pancreatic carcinoma cells. Since flow cytometry revealed absence of CAR expression, but the presence of at least one of both αv integrins on the pancreatic carcinoma cells, a second way of targeting was investigated using a genetically modified Ad vector which has an RGD (Arg-Gly-Asp)-containing peptide inserted into the HI-loop of the fiber knob. This RGD targeted Ad (AdlucRGD) revealed efficient CAR-independent infection by allowing binding to cellular integrins resulting in a dramatic enhancement of gene transfer. These findings have direct relevance for Ad-vector based gene therapy strategies for pancreatic carcinoma. Gene Therapy (2001) **8,** 969–976.

Keywords: *pancreatic carcinoma; primary tumor cells; adenovirus vector; targeting; gene therapy*

Introduction

Pancreatic cancer is highly aggressive and ranks fifth among malignancy-associated deaths. Prognosis remains dismal because diagnosis of pancreatic cancer is made late in the clinical course of the disease. Currently, there is no effective treatment for this disease: resection is only available to a small fraction of patients presenting with locally confined tumor.[1] Chemotherapy and radiation also have limited effects on patient survival. Adjuvant combined radiochemotherapy might potentially improve survival and can be considered in unresectable, locally advanced disease. However, the role of chemotherapy in advanced disease is exclusively palliative.[1] Therefore, development of new therapeutic modalities such as gene therapy are necessary to improve patient outcome and serve as a more effective treatment for pancreatic cancer.

Adenoviral vectors have been used for both *in vitro* and *in vivo* gene delivery of pancreatic cancer, mainly because of their ability to infect both dividing pancreatic cancer cells, as well as nondividing tumor cells. Another advantage is that the techniques to produce high-titered preparations of adenovirus vectors are relatively simple. Furthermore, phase I clinical trials employing adenovirus vectors have been started already for pancreatic cancer.[2] As observed for other tumor tissue types, a major concern associated with using adenovirus vectors in pancreatic cancer is the relatively limited infection efficiencies achieved *in vitro*.[3] Furthermore, *in vivo* gene delivery may be limited by other factors, such as vector's access to target cells through local dissemination or through penetration of vessel walls.

Studies on adenoviral entry into host cells have revealed that two cell surface events, attachment and internalization, are required for an adenovirus to enter a cell.[4] The viral fiber protein will first attach to the CAR (Coxsackie and adenovirus receptor) on the surface of a host cell.[5] The virion then enters the cell through the interaction of its penton base with the αvβ3 and αvβ5 integrins on the host cell surface.[4] Expression of these cell surface markers and their correlation with the efficiency of adenovirus-mediated gene transfer have revealed that the presence of integrins αvβ3,[6] αvβ5[7] and CAR[8,9] are important for an efficient gene transfer and efficacy of

Correspondence: DT Curiel, Division of Human Gene Therapy, Dept of Medicine, Pathology, Surgery and Gene Therapy Center, The University of Alabama at Birmingham, 1824 6th Avenue South, 620 Lurleen Wallace Tumor Institute, Birmingham AL 35294–3300, USA
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infection. Recently, a relative lack of CAR on both neoplastic[10] and non-neoplastic[11] tissues has been implicated as a limiting factor in succesful adenovirus gene transfer.

To overcome the problems associated with *in vitro* and *in vivo* gene delivery to tumor cells, retargeting of adenovirus has been endeavored as a means to improve its specificity and efficacy. Retargeting allows adenovirus to bind to alternative cellular receptors, resulting in CAR-independent infection. One approach is based on immunological retargeting, which uses an antifiber antibody or antibody fragment that is chemically conjugated to either a cell-specific ligand (receptor) or antireceptor antibody. Using this conjugate approach, successful CAR-independent gene transfer has been achieved by targeting viral infection *in vitro* to several cellular receptors including integrins,[12] the folate receptor,[13] the basic fibroblast growth factor (FGF2) receptor[14] and the epidermal growth factor (EGF) receptor.[10] Retargeting of adenovirus to EGFR was recently shown to enhance gene transfer in primary, low-passage glioma tumor cells as well as in squamous cell carcinoma of the head and neck, which suggests further clinical relevance for retargeting.[10,15]

A potential disadvantage of this conjugate approach may be the introduction of a degree of complexity to the vector system and the concerns regarding the stability of the virus–conjugate complex under *in vivo* conditions such as systemic administration. Therefore, a genetically modified targeted viral particle might be a more attractive vector candidate for clinical application. Insertion of an Arg-Gly-Asp (RGD) motif into the HI-loop of the adenoviral fiber knob results in efficient CAR-independent infection by allowing binding of the virus to cellular integrins.[16,17] The vector containing this fiber, AdlucRGD, achieved dramatically augmented gene delivery to several cell types, both *in vitro*[17] and *in vivo*.[18]

To direct Ad gene delivery specifically to pancreatic carcinoma cells we chose two approaches. First, we have explored the utility of a bispecific fusion protein formed by a recombinant soluble form of truncated CAR (sCAR) genetically fused with human EGF (sCAR-EGF) to target Ad infection to the EGF receptor expressed on established and primary human pancreatic carcinoma cells. An approach based on employment of soluble viral receptor-EGF fusion proteins has ben originally established for targeting retroviral infection to specific cell types.[19,20] We recently showed that sCAR-EGF fusion protein possesses the ability to effectively retarget the vector via the EGF receptor with enhancement of gene transfer efficiency.[21] Second, we have shown that recombinant Ad vector containing fibers with RGD motif in the HI loop is capable of augmenting gene delivery to established and primary pancreatic carcinoma cells via a CAR-independent cell entry mechanism using the integrins as receptor. These findings have direct relevance for Ad vector-based gene therapy strategies for pancreatic carcinoma.

Results

Detection of CAR-receptors on primary and established human pancreatic carcinoma cells

It has been shown that human pancreatic carcinoma cell lines are quite refractory to infection of adenovirus vectors due to the low expression level of cell surface molecules involved in adenovirus infection, ie αv-integrins and the recently identified CAR.[3] Therefore, we decided to analyze four established human pancreatic carcinoma cell lines, and two primary human pancreatic carcinoma cells, for cell surface CAR expression by indirect immunofluorescence using an anti-human murine polyclonal serum to CAR. As shown in Figure 1a, both the four pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T or MIA PaCa-2), as well as the two primary pancreatic carcinoma cells (p6.3 and p10.5) displayed very low levels of cell surface CAR expression. This CAR deficiency strongly suggests a low level of adenovirus-directed gene transfer to these primary and established pancreatic carcinoma cells will result if Ad5 vectors with unmodified

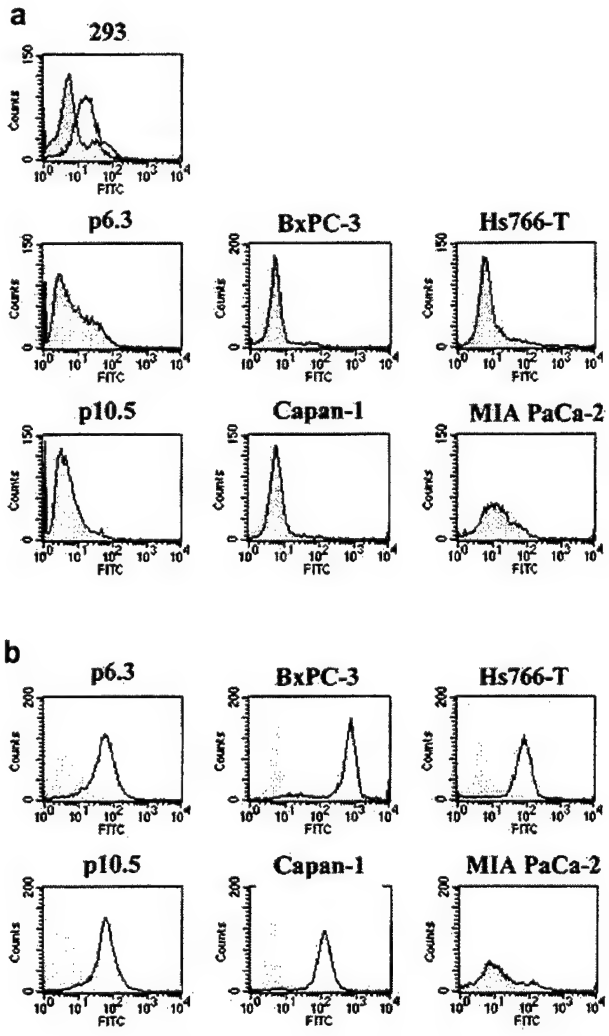

Figure 1 Expression of CAR and EGFR in human pancreatic carcinoma cells. (a) Indirect flow cytofluorometry is shown for the expression of CAR (black line) in the primary human pancreatic carcinoma cells (p6.3 and p10.5), in the established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2) and in the positive control cell line (293). Normal mouse serum is used as a control (blue peak). (b) Indirect flow cytofluorometry is shown for the expression of EGFR (black line) in the primary human pancreatic carcinoma cells (p6.3 and p10.5) and in established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2). Normal mouse IgG is used as a control (blue peak).

fiber knobs are used (see below), as has been shown for head and neck cancer[22] and for ovarian cancer.[23] On this basis we considered alternative receptors that might be upregulated in pancreatic carcinoma cells and which could be exploited for targeting.

Expression of EGF-receptors on primary and established pancreatic carcinoma cells

The low levels of the native adenovirus receptor CAR in the two primary pancreatic carcinoma cells and the four pancreatic carcinoma cell lines, predicted that they would be refractory to adenovirus infection. Thus, we sought alternative receptors to exploit for targeting. In this regard, the epidermal growth factor (EGF) receptor has shown to be upregulated in a number of human tumors, ie squamous cell carcinoma of the head and neck[15] and in human glioma.[10] Therefore, we evaluated our established pancreatic carcinoma cell lines and the primary pancreatic carcinoma cells for this receptor by flow cytometry using an anti-EGF receptor (EGFR) antibody. Greater than 85% of the cells in three out of four established pancreatic carcinoma cell lines and in both primary pancreatic carcinoma cells analyzed expressed high levels of EGFR (Figure 1b). Based on the observed differences in CAR and EGFR expression levels in pancreatic carcinoma cells (Figure 1), we hypothesized that adenovirus infection would be more efficient if the vector was redirected to EGFR.

EGFR-retargeted gene delivery to primary and established pancreatic carcinoma cells

The high EGFR levels on both the established, as well as the primary, pancreatic carcinoma cells offer a potential target for a modified adenovirus vector that is capable of utilizing this receptor. To demonstrate the utility of EGFR retargeting we fused a recombinant form of truncated CAR (sCAR) with human EGF as a targeting ligand (sCAR-EGF) and investigated its ability to target Ad infection to the EGF receptor overexpressed on pancreatic carcinoma cells. In a previous study Dmitriev *et al*[21] have shown that sCAR-EGF protein is capable of binding to Ad virions and directing them to EGFR. The sCAR-EGF protein was titered against Ad to ascertain the optimal ratio of targeting protein to virus as measured by improvements in gene transfer (results not shown). To demonstrate EGFR retargeting, both the established as well as the primary pancreatic carcinoma cells were infected with either native AdCMVLuc or sCAR-EGF-complexed AdCMVLuc or sCAR-6His-complexed AdCMVLuc. sCAR-6His serves as a relevant control protein to show that sCAR-EGF promoted gene transfer occurs by an EGFR-specific mechanism and no enhancement is observed in cells exposed to AdCMVLuc complexed with sCAR-6His, as shown earlier.[21] Forty-eight hours after infection, cells were lysed and luciferase activity was measured. As shown in Figure 2, compared with AdCMVLuc alone or with AdCMVLuc complexed with sCAR-6His, AdCMVLuc complexed with sCAR-EGF targeting protein mediated 1.5-, two-, three- and five-fold enhancement of luciferase expression in p10.5, p6.3, Capan-1 and BxPC-3 cells, respectively, both using an MOI of 10 and 100. In Hs-766T cells a 1.5-fold enhancement of luciferase expression is only seen at an MOI of 10. Although the expression level of the EGF receptors on MIA PaCa-2 cells appeared to be very low

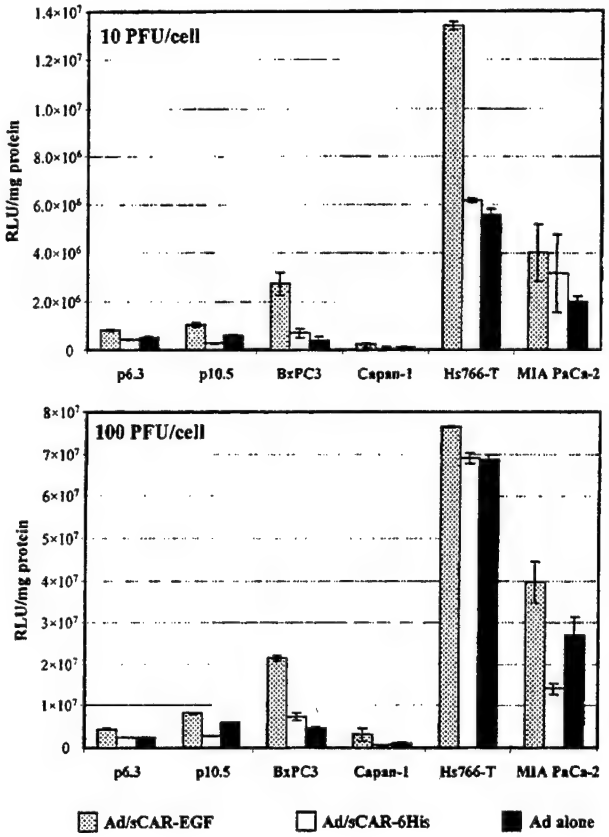

Figure 2 Comparison of EGFR-targeted Ad gene transfer to human pancreatic carcinoma cells. An amount of 3 × 10⁸ p.f.u. of AdCMVLuc was preincubated with either 15 μg of targeting sCAR-EGF protein (Ad/sCAR-EGF ▩) or with 12 μg of sCAR-6His as a control (Ad/sCAR-6His □) or with PBS (Ad alone ■) before incubation with cells. Then monolayers of primary pancreatic carcinoma cells (p6.3 and p10.5), as well as established pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs 766-T and MIA PaCa-2) were exposed to Ad or Ad/sCAR-ligand complexes at MOI (10 or 100 p.f.u./cell) for 1 h at 37°C. Infection medium was changed for complete medium and the cells were incubated for 48 h at 37°C. The cells were lysed, the protein concentration and luciferase activities of the lysates were determined. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically. The results are shown as the mean of multiple assays. Each point represents the mean ± standard deviation of two determinations. Error bars, s.e.

(Figure 1b), still a 1.5-fold enhancement of luciferase expression is observed using AdCMVLuc complexed with sCAR-EGF (Figure 2). These results demonstrated that sCAR-EGF targeting protein enables retargeting of Ad vector with several-fold enhancement of gene transfer efficiency specifically to EGFR-positive pancreatic carcinoma cells (both primary and established cell lines). The sCAR-EGF promoted gene transfer occurs by an EGFR-specific mechanism, since no significant enhancement was observed in cells exposed to AdCMVLuc complexed with sCAR-6His (untargeted Ad). Furthermore, the specificity of sCAR-EGF-mediated Ad-targeting was illustrated by failure of the sCAR-EGF to enhance Ad-based gene transfer to EGFR-negative human mammary gland (MDA-MB-453) cells (results not shown; Ref. 21).

Expression of αvβ3 and αvβ5 integrins on primary and established pancreatic carcinoma cells

Because EGFR retargeting by sCAR-EGF enhanced adenovirus gene transfer with variable efficiency and

with relatively low retargeting indexes (range of enhancement of 1.5- to five-fold, Figure 2), as compared with human squamous carcinoma (SCC-4) cells and human epidermoid carcinoma (A-431) cells,[21] we looked for targeting via other cellular receptors. It has been shown that insertion of an Arg-Gly-Asp (RGD) motif in the HI-loop of the Ad5 knob domain resulted in a viral fiber protein which results in efficient CAR-independent infection by allowing binding of Ad to cellular integrins.[17] The vector containing this fiber (AdlucRGD) indeed achieved augmented gene delivery to several cell types by interaction to cellular αv integrins and thus allowing CAR-independent infection.[17,18] We already determined the low level of expression of the native CAR receptor on the surface of established and primary pancreatic carcinoma cells (Figure 1). Therefore, to assess if a genetically modified Ad containing an RGD motif can efficiently enter the pancreatic carcinoma cells, we ascertained the level of expression of both αvβ3 and αvβ5 integrins on these tumor cells. Flow cytometry was performed to detect expression of both αvβ3 and αvβ5 integrins on established and primary pancreatic carcinoma cells by indirect immunofluorescence using LM609 and P1F6 mAbs, respectively. As shown in Figure 3, expression of αvβ3 integrin is absent in p6.3, BxPC-3 and Capan-1 cells, while a low to moderate αvβ3 expression is seen in p10.5, Hs766-T, MIA PaCa-2 cells. Integrin αvβ5, on the other hand, is present on all pancreatic cancer cells: p6.3, p10.5 and Capan-1 cells express high levels of αvβ5, while the other pancreatic carcinoma cell lines (BxPC-3, Hs766-T, MIA PaCa-2) express moderate levels of this integrin. Thus, the presence of one or both of the αv integrins on the established and primary pancreatic carcinoma cells should allow CAR-independent gene transfer by AdlucRGD.

Integrin targeted gene delivery to primary and established pancreatic carcinoma cells

Our next goal was to examine whether introduction of the RGD motif in the fiber of AdlucRGD resulted in an enhancement of this virus' ability to infect established and primary pancreatic carcinoma cells. Therefore, AdlucRGD was utilized for an assay based on competitive inhibition of Ad-mediated gene delivery by recombinant Ad5 fiber knob protein, known to efficiently block virus binding to CAR.[17] To demonstrate CAR-independent cell entry by AdlucRGD, established and primary pancreatic carcinoma cells were infected with various MOIs with either native AdCMVLuc or integrin-retargeted AdlucRGD in the presence or absence of blocking knob protein. Forty-eight hours after the infection, cells were lysed and luciferase activity was measured. As shown in Figure 4a in both established as well as primary pancreatic carcinoma cells, striking differences between the infection profiles were demonstrated by these two viruses at each MOI. Luciferase expression in the AdlucRGD-infected primary pancreatic carcinoma cells was 100- to 500-fold higher than in the cells infected with AdCMVLuc. Even in the established pancreatic carcinoma cell lines the difference in infection efficiencies demonstrated by these two viral vectors was still between 10- and 100-fold. Of note, comparison of luciferase expression upon infection of 293 cells (high CAR expression) with AdCMVLuc *versus* AdlucRGD did not reveal a substantial enhancement of gene transfer with the AdlucRGD-targeted virus (results not shown).

Furthermore, AdCMVLuc-mediated infection in the presence of Ad5 fiber knob protein revealed a dramatic inhibition. The knob blocked between 55% and 95% of the gene transfer at MOIs of 10 and 100 p.f.u. for primary pancreatic carcinoma cells, while this protein blocked between 40% and 90% of the gene transfer at MOIs of 1, 10 and 100 p.f.u. for established pancreatic carcinoma cell line (Figure 4b). Most importantly, for the primary p10.5 pancreatic carcinoma cells as well as the established Capan-1, Hs766-T and MIA PaCA-2 pancreatic cell lines, recombinant knob protein did not reveal any significant inhibition effect on the levels of luciferase expression directed by AdlucRGD (Figure 4b). Strong inhibition by the fiber knob on AdCMVLuc-mediated luciferase expression suggests that the fiber–CAR interaction is the only pathway this virus can use to infect pancreatic carcinoma cells.

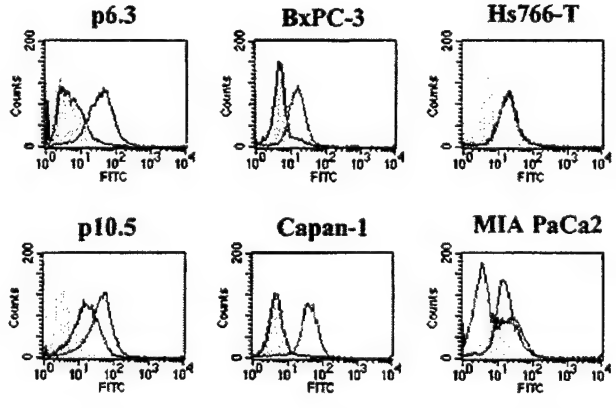

Figure 3 Expression of αv-integrins in human pancreatic carcinoma cells. Indirect flow cytofluorometry is shown for the expression of αvβ3-integrins (black line) and αvβ5-integrins (red line) in primary human pancreatic carcinoma cells (p6.3 and p10.5) and in established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2). Normal mouse IgG is used as a control (blue peak).

Gene delivery of an integrin-targeted Ad vector increases the frequency of infection of pancreatic carcinoma cells

The previous experiments demonstrated that adenovirus infection of both primary and established pancreatic carcinoma cells can be redirected to the αv-integrins via a CAR-independent pathway, resulting in enhanced reporter gene expression. This observed enhancement of gene transfer with the AdlucRGD virus could have arisen from either a few transduced cells exhibiting more abundant gene expression because an increased number of viruses have infected these cells or from a greater number of pancreatic carcinoma cells that may have been rendered susceptible to infection because of the high amount of expressed αv-integrins on the cell surface. To identify the nature of the enhanced gene expression CAR-independent retargeting was performed using an adenovirus that expressed the green fluorescent protein (GFP) reporter gene. Both primary, as well as established, pancreatic carcinoma cells were infected with either native AdGFP or with the genetically modified AdGFP-RGD at an MOI of 10, 100 and 1000 per cell during 48 h and the number of infected cells were monitored by fluorescent

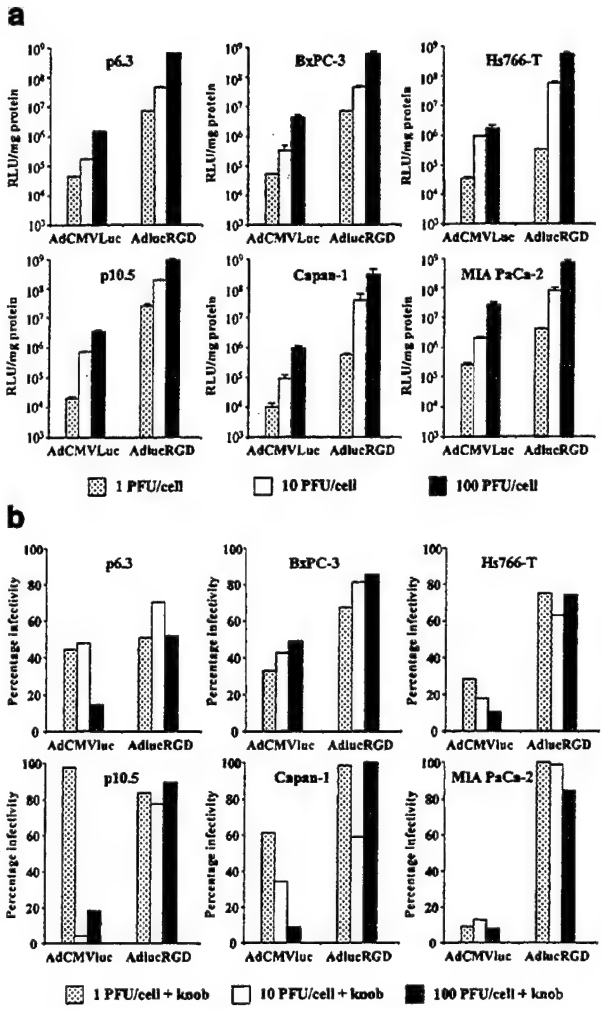

Figure 4 Comparison of Ad gene transfer efficiencies of AdCMVLuc and AdlucRGD to the human pancreatic carcinoma cells. (a) Primary human pancreatic carcinoma cells: p6.3 and p10.5 and established human pancreatic carcinoma cell lines BxPC-3, Capan-1, Hs766-T and MIA PaCa-2 were infected with Ad5CMVLuc or AdlucRGD at an MOI of 1 (▨), 10 (□), and 100 (■) p.f.u./cell. Infection medium was changed for complete medium 1 h after infection and the cells were incubated for 28 h at 37°C. The cells were lysed, the protein concentration and luciferase activity of the lysates were determined. The relative light units (RLU) of luciferase/milligram of total cellular protein are shown graphically. The results are shown as the mean of multiple assays. Each point represents the mean ± standard deviation of two determinations. (b) Primary human pancreatic carcinoma cells: p6.3 and p10.5 and established human pancreatic carcinoma cell lines BxPC-3, Capan-1, Hs766-T and MIA PaCa-2 were preincubated in PBS or PBS containing recombinant Ad5 fiber knob protein for 30 min at 37°C. AdCMVLuc or AdlucRGD were added to the cells at an MOI of 1 (▨), 10 (□), and 100 (■) p.f.u./cell for 1 h, then media was changed and the cells were incubated at 37°C. At 48 h after infection the cells were lysed, the protein concentration and luciferase activity of the lysates were determined. The percentages of luciferase activity detected in the cells infected in the presence of knob calculated with regard to luciferase activity detected in the cells infected in the absence of knob (=100%, not shown on the graph) are depicted graphically.

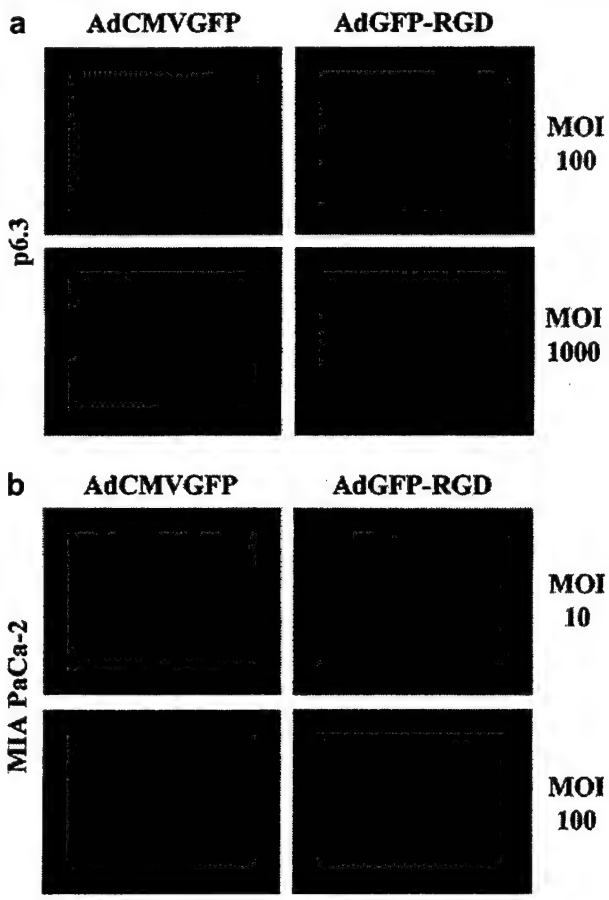

Figure 5 Analysis of the number of infected pancreatic carcinoma cells using AdCMVGFP and AdGFP-RGD. (a) Infection of the primary pancreatic carcinoma cells (p6.3) was peformed at an MOI of 100 or 1000 v.p./cell and (b) the established pancreatic carcinoma cell line (MIA PaCa-2) was infected at an MOI of 10 or 100 p.v./cell. Forty-eight hours after infection the percentage of infected cells was determined using the the fluorescence microscope.

microscopy. Figure 5 reveals the results of retargeting via the αv-integrins of two representative pancreatic carcinoma cells: the p6.3 primary pancreatic carcinoma cells and the established pancreatic carcinoma cell line MIA PaCa-2. Using the native AdCMVGFP virus only a low amount of pancreatic carcinoma cells were infected, while retargeting via the αv-integrins by the CAR-independent route using AdGFP-RGD resulted in increased numbers of cells that were positive for green fluorescent protein expression both for the p6.3 primary cells (Figure 5a) as well as for the MIA PaCa-2 established pancreatic carcinoma cell line (Figure 5b). As a control, infection of 293 cells (high CAR expression) with AdCMVGFP and AdGFP-RGD viruses revealed a significant lower percentage of gene transfers with AdGFP-RGD compared with untargeted AdCMVGFP (data not shown). These data indicate that retargeting of the adenovirus to the αv-integrins via a CAR-independent cell entry increased the number of infected pancreatic carcinoma cells instead of showing more abundant gene expression in the infected cells.

Discussion

Human pancreatic carcinoma cell lines have been shown to be relatively resistant to adenovirus mediated gene transfer. These unmodified, first-generation adenovirus vectors have failed to deliver genes at an efficiency that

would be therapeutic in the context of human pancreatic cancer. Pearson *et al*[3] revealed that the low expression levels of the integrins αvβ3, αvβ5, α5, β1 and CAR will limit adenovirus-mediated gene transfer in both lung and pancreatic cancer cell lines and their results were confirmed in this study. Importantly, the two primary (passage number lower than five) human pancreatic carcinoma cells (p6.3 and p10.5) used in this study were revealed to be refractory to adenoviral infection to the same amount as the established, highly passaged pancreatic carcinoma cell lines. For this reason, in this report, we have demonstrated two ways of retargeting of adenovirus vectors as a means to enhance gene transfer efficiency to both primary and established human pancreatic carcinoma cells. First, we showed that EGFR-retargeted entry pathway using the sCAR-EGF protein, which binds to Ad virions and redirects them to EGFR,[21] was able to enhance the gene transfer efficiency of pancreatic carcinoma cells between 1.5- and five-fold, most likely via a non-CAR pathway. Increase of gene transfer by retargeting to EGFR is explained by flow cytometry analyses which revealed that EGFR is overexpressed in both primary, as well as established, pancreatic carcinoma cells. It is very likely that redirecting the virus to the highly expressed EGFR molecule increased the overall number of cells infected, thereby leading to an enhanced gene transfer, as has been shown for head and neck cancer cells.[15] Of importance, immunohistochemical staining of normal and adenocarcinoma pancreatic tissue slides (resection material from patients) with anti-EGFR antibody revealed a moderate to high expression level of EGFR molecules on most (adeno)carcinoma cells of the pancreas compared with the surrounding normal pancreas or normal liver cells (unpublished observations). Therefore, it is not likely that EGFR-targeted Ad vectors will cause any significant toxicity to non-diseased pancreatic or liver tissue. The increase in gene transfer shown here for pancreatic carcinoma is significantly lower than seen by Dmitriev *et al*[21] who revealed that Ad encoding luciferase complexed with sCAR-EGF targeting protein mediated a much higher enhancement of luciferase gene expression in EGFR-positive SKOV3.ip1 (human ovarian carcinoma) cells, EGFR-positive SCC-4 (human squamous carcinoma) cells and EGFR-positive A-431 (human epidermoid carcinoma) cells. This difference is most likely explained by the higher number and/or expression level of the EGF-receptors on these SKVO3.ip1, SCC-4 and A-431 cells compared with the primary and established pancreatic carcinoma cells. Despite the conceptual gains realised by this conjugate approach, this 'two-component' strategy introduces a degree of complexity to the vector system. Moreover, it raises concerns regarding the stability of the virus–conjugate complex under certain *in vivo* conditions.[18] Therefore, we decided to exploit another targeting approach.

Genetically modified vectors containing short peptide targeting sequences at the C-terminus of the adenoviral knob domain (which binds to CAR) have been produced, which revealed expanded viral tropism *in vitro* by targeting to, for example, integrins.[24] However, the C-terminus of the knob domain is located at the base which is not an ideal position to interact efficiently with cellular receptors. Recently it has been reported that the use of an alternate region of the knob domain, the HI-loop, is a more rational site for inserting targeting motifs.[16] This region is not directly involved in trimerization, it contains mostly hydrophilic amino acids and is of different length in different Ad serotypes. Furthermore, the HI-loop is flexible and is exposed on the exterior of the knob. Insertion of an RGD motif in this region resulted in a viral fiber protein which results in efficient CAR-independent infection by allowing binding of Ad to cellular integrins.[17] The use of RGD-modified viral vectors (AdlucRGD and AdGFP-RGD) in this study is based on flow cytometry data using anti-CAR and anti-αvβ3/αvβ5 antibodies which revealed no expression of CAR, but shows the presence of at least one of the αv-integrins on the surface of the primary and established pancreatic carcinoma cells. The resulting modified virus (AdlucRGD) revealed a dramatic increase in gene transfer efficiency of both primary and established pancreatic carcinoma cells.[16,17] The absence of CAR expression and the presence of the αv-integrins on the surface of these cells explains the enhanced luciferase expression of AdlucRGD compared with parental AdCMVLuc. Furthermore, the ability of the knob protein to block infection using parental AdCMVLuc as well as the lack of inhibition in the presence of this knob protein on the levels of luciferase expression directed by AdlucRGD, clearly demonstrated that RGD-modified Ad has enhanced binding to pancreatic carcinoma cells lacking CAR, leading to enhanced gene expression. The observation that knob inhibited luciferase expression of AdlucRGD in p6.3 cells and to a lesser extent in BxPC-3 cells, suggests that in these particular pancreatic carcinoma cells either knob may interfere with binding of AdlucRGD to the integrins or that a minor fraction of AdlucRGD enters these cells via the CAR.

Of note, infection of pancreatic carcinoma cells with AdGFP-RGD revealed that the number of infected pancreatic carcinoma cells has been increased rather than a few cells exhibiting more abundant gene expression as compared with parental AdGFP. The importance of both latter findings lay in the fact that fewer AdRGD virus particles need to be administered *in vivo* to obtain the same therapeutic effect, thereby decreasing the vector-related toxicity. Importantly, although Ad5lucRGD has expanded tropism,[18] this genetically modified virus is not tumor-specific. However, in a study where both primary ovarian tumor explants, as well as nontumor mesothelial tissue samples, from patients were infected with Adluc and AdlucRGD, the mesothelial tissue samples expressed low luciferase activity both with the Ad5lucRGD vector as with AdCMVLuc.[23] Therefore, studies will be initated to assess the efficacy of infection of normal pancreatic epithelial cells obtained from human resection material with Ad targeted to EGFR, as well as Ad targeted to integrins (AdlucRGD).

To our knowledge this is the first study where enhanced gene transfer by EGFR, as well as integrin-targeted adenovirus vectors has been demonstrated in primary pancreatic carcinoma cells. Of note, the infection conditions were selected for representing a high level of stringency that a gene transfer vector would have to overcome in the clinical context. The observed level of enhancement as seen with the EGFR-retargeted vector to a minor extent and with the RGD-modified Ad vector to a major extent, would thus support the use of these vectors in human gene therapy clinical trials for pancreatic carcinoma. Thus, this study seeks to validate a strategy

that will address a critical shortcoming in cancer gene therapy. The key finding in this study is that gene transfer to both primary pancreatic cancer cells, as well as established pancreatic carcinoma cell lines, is significantly enhanced by utilizing an RGD-modified retargeted vector. As integrins have been frequently shown to be overexpressed by various epithelial tumors, as described for head and neck cancer[22] and ovarian cancer,[23] our novel vector strategy could potentially be exploited in the context of pancreatic cancer. Most importantly, the levels of gene transfer of the RGD-modified adenovirus in surrounding nontumor pancreatic epithelial cells provides a rationale for further studies with this targeted vector in preclinical efficacy studies that would lead to human clinical trials.

Materials and methods

Tumor cells

The established human pancreatic carcinoma cell lines (BxPC-3, Capan-1, Hs766-T and MIA PaCa-2; >20 passages) were purchased from Boehringer Ingelheim, Belgium. These cells were cultured in Dulbecco's minimal essential medium (DMEM) (Mediatech, Herndon, VA, USA) with 10% fetal bovine serum (FBS) (Summit Biotechnology, Ft Collins, CO,USA), 1% L-glutamine and 1% penicillin/streptomycin (Gibco BRL, Life Technologies, Rockville, MD, USA). The primary human pancreatic carcinoma cells (p6.3 and p10.5; <5 passages) were obtained from Dr E Jaffee, Johns Hopkins University School of Medicine, Baltimore, MD, USA. All cell lines were cultured at 37°C in 5% carbon dioxide atmosphere.

Viruses, antibodies and recombinant proteins

The E1-, E3-deleted adenovirus vector expressing the firefly luciferase from the cytomegalovirus (CMV) immediate–early promoter, AdCMVLuc[25] was obtained from Robert Gerard (University of Leuven, Leuven, Belgium). Ad vector AdlucRGD, containing recombinant fiber-RGD protein and expressing firefly luciferase was generated by transfection of 293 cells with *Pac*I-digested pVK703.[18] Ad vector AdCMVGFP, encoding green fluorescent protein, and AdGFP-RGD, containing recombinant fiber-RGD protein and expressing GFP were obtained from Dr M Parameshwar (University of Alabama at Birmingham, AL, USA). Viruses were propagated and plaque-titered on the permissive cell line 293 and purified by double cesium chloride gradients.[26] Virus preparations were dialyzed against phosphate-buffered saline (PBS), aliquoted, and stored at –80°C. Titers were determined using standard plaque assays and the number of viral particles was determined by measuring the optical density at 260 nm. For AdCMVLuc: 4.2×10^{11} p.f.u./ml and 8.1×10^{12} v.p./ml (v.p. to p.f.u. ratio: 19.3) and for AdlucRGD: 5.6×10^{10} p.f.u./ml and 1.7×10^{11} v.p./ml (v.p. to p.f.u. ratio: 32.9).

Murine polyclonal serum to baculovirus-produced human soluble CAR protein were generated at the University of Alabama at Birmingham, Hybridoma Core Facility. Murine mAb 425 to human EGFR was a generous gift from Zenon Steplewski (Thomas Jefferson University, Philadelphia, PA, USA) and was described earlier.[27] Murine mAb LM609 to αvβ3 integrin and P1F6 to αvβ5 integrin were purchased from Chemicon (Temecula, CA, USA). Recombinant fiber knob from Ad5 was obtained from Dr V Krasnykh (University of Alabama at Birmingham). Recombinant sCAR-6His and sCAR-EGF proteins were constructed as described.[21]

Flow cytometry

Confluent cells were released with versene or cell dissociation buffer (Gibco BRL, Life Technologies) or by trypsinizing of the cells using 0.05% trypsin/0.53 mM EDTA for 3 min or less (2 ml per T75 flask). The trypsinized cells were quenched with 10-fold volume of cold DMEM-medium containing 10% fetal bovine serum and pelleted at 1200 r.p.m. for 5 min. Cells were resuspended in cold PBS with 1% bovine serum albumin (BSA) and counted. Cells were spinned (1200 r.p.m. for 5 min) and aliquoted in PBS + BSA at 2×10^6 cells/ml. Cells (2×10^5) were incubated with either mAb 425 (5 μg/ml) (anti-EGFR) or with murine anti-CAR serum (1:250) or with murine mAb LM609 (anti-αvβ3) or with mAb P1F6 (anti-αvβ5) for 1 h at 4°C. A normal murine serum and control IgG were used as a negative control. Cells were then washed with buffer and incubated with secondary FITC-labeled goat anti-mouse immunoglobulin G (Jackson, West Grove, PA, USA) at a concentration of 5 μg/ml for 1 h at 4°C. After washing, 10^4 cells per sample were analyzed using flow cytometry performed at the University of Alabama at Birmingham FACS Core Facility. Data were expressed as the geometric mean fluorescence intensity of the entire gated population. The positive population cells was determined by gating the right-hand tail of the distribution of the negative control sample for each individual cell line at 1%. This gate setting was then used to determine the percentage of CAR-, αvβ3-, αvβ5- or EGFR-positive cells in each individual cell line.

Adenovirus vector-mediated gene transfer

To assess native or EGFR-retargeted adenovirus infection efficiency, 5×10^4 cells were plated in 24-well plates and allowed to adhere overnight at 37°C. An amount of 3×10^8 p.f.u. of AdCMVLuc was preincubated with either 15 μg of sCAR-EGF protein or 12 μg of sCAR-6His as a control or with PBS, before incubation with cells for 30 min at room temperature. Then monolayers of pancreatic carcinoma cells were exposed to Ad/sCAR–ligand complexes and Ad without ligand complexes at various MOIs (10 and 100 p.f.u. cell) cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media.

To assess native or RGD-modified adenovirus efficiency, 5×10^4 cells were plated in 24-well plates and allowed to adhere overnight at 37°C. To demonstrate the specificity of infection, half of the cells were blocked with recombinant Ad5 knob protein (10–15 μg/well, diluted in PBS) for 30 min at 37°C and the other half were incubated with PBS as a control. Subsequently, both the blocked cells, as well as the unblocked cells, were infected with native AdCMVLuc or AdlucRGD at various MOIs (1, 10 and 100) per cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media.

Cell lysates were assayed for luciferase expression 48 h after infection in a Berthold Luminometer using the Luciferase Assay System (Promega, Madison, WI, USA), and the protein concentration was determined using the

Pierce Protein Assay according to the manufacturer's protocols.

To evaluate the number of transfected cells, 5×10^4 cells were plated in 24-well plates and allowed to adhere overnight at 37°C. The cells were infected with AdCMVGFP and AdGFP-RGD at various MOIs (10, 100 and 1000) per cell for 1 h at 37°C. The virus was removed and the cells were incubated for 48 h in complete media. Subsequently, the media was removed and PBS was added to the wells and the percentage of infected cells were visualized under a fluorescence microscope.

Acknowledgements

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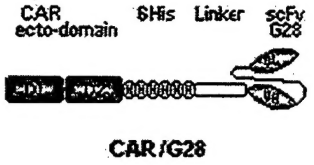

Figure 1 Structure of CAR/G28 fusion protein. The fusion protein consists of an extracellular portion of human CAR (domains D1 + D2) at the N-terminus connected to anti human CD40 scFv G28 via a short peptide linker (PSASASASAPGS) preceded by a 6 histidine (6His) purification tag. CAR binds to Ad5 fiber knob, whereas G28 binds to CD40 present on DCs, thus facilitating Ad viral docking to DCs.

bispecific molecule due to the inherent variability of the technique. A product intended for clinical use should have a strictly defined and reproducible composition. Recombinant proteins achieve this by rational design. We have recently described an approach of bispecific adaptor-based Ad targeting with a recombinant protein consisting of an extracellular portion of Ad receptor CAR fused to epidermal growth factor (EGF).[11] In that study, the CAR/EGF fusion protein specifically directed Ad infection to EGF receptor expressing cells that lacked expression of CAR. In the present study, our aim was to create a fusion protein between CAR and a single chain antibody against human CD40 (Figure 1). We hypothesized that such a recombinant CAR-anti-CD40scFv protein would facilitate efficient, specific DCs transduction with Ad.

The CAR/G28 fusion protein was generated by the following scheme. Initially, the extracellular domain of human CD40 (hCD40ecto, including amino acids 21–193, numbered according to Ref. 12) was produced in a prokaryotic expression system and purified. The anti-CD40 single chain Fv cDNA was generated from the G28-5 hybridoma cell line.[13] The hCD40ecto was then used to screen for CD40-specific scFv. The G28-5 scFv cDNA was linked to that of the CAR ecto-domain replacing the EGF portion in the CAR/EGF fusion protein.[11] Finally, the CAR/G28 fusion protein was produced using recombinant baculovirus, purified and characterized.

Recombinant hCD40ecto was produced in a prokaryotic expression system to facilitate anti-CD40 scFv and CAR/scFv isolation and validation. The hCD40ecto gene fragment was PCR-amplified from a cDNA clone generously provided by Dr I Stamenkovic (Molecular Pathol-

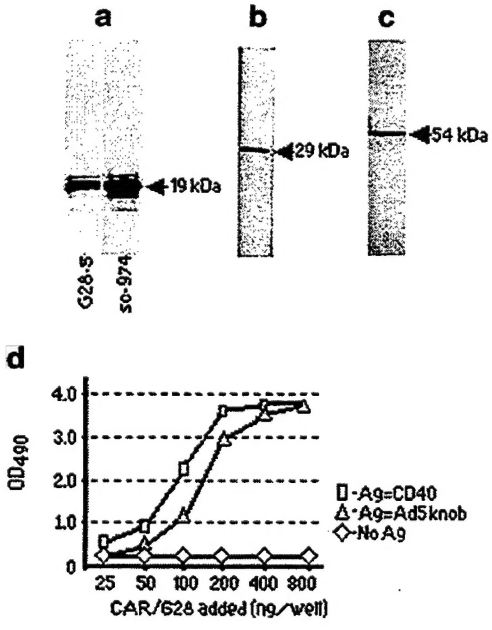

Figure 2 Fusion proteins production and characterization. (a) Production of ecto-domain of human CD40. To produce the hCD40ecto as a 6His-tagged recombinant protein, the corresponding gene fragment was PCR-amplified from a cDNA clone and ligated into pET21a expression vector (Novagen, Madison, WI, USA) using BamHI and NotI restriction sites. For the amplification, a pair of primers 5′ CTT GGA TCC GAA CCA CCC ACT GCA TGC AGA GAA A and 5′ ATT GCG GCC GCT CTC AGC CGA TCC TGG GGA CCA was used. The assembled plasmid was introduced into BL21(DE) E. coli cells and the gene expression was induced with IPTG. Produced protein was purified from cell lysate by immobilized metal affinity chromatography (IMAC) on Ni-NTA (Qiagen, Valencia, CA, USA). The purified protein (100 ng/lane) was separated on a 12% polyacrylamide-SDS gel (PAAG-SDS) followed by electrotransfer to a PVDF membrane. The membranes were probed with the hybridoma supernatant of mouse mAb G28-5 (ATCC No. HB9110) and with rabbit antibody sc-974 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), both specific for human CD40. (b) Construction of scFv G28-5 against human CD40. The G28-5 scFv was generated from a corresponding hybridoma using published sequences (Genebank accession numbers are AF013577 for VH and AF013576 for VL) for primer design. Hybridoma cells grown from a fresh clone were harvested and mRNA was isolated from 4×10^6 cells using an Oligotex Direct mRNA Micro Kit from Qiagen. Complimentary DNA was synthesized from this mRNA using an Omniscript reverse transcription kit from Qiagen and an Oligo(dT)15 primer from Promega (Madison, WI, USA). The VH and VL fragments were amplified from the cDNA and cloned into pSEX81 phagemid vector. Two pairs of primers G28HF: 5′ GAT ATA CAG CTT CAG CAG TC and Bi4: 5′ CCA GGG GCC AGT GGA TAG ACA AGC TTG GGT GTC GTT TT and G28LF: 5′ GAT GCT GTG ATG ACC CAA AAT and G28LR: 5′ GGA TAC AGT TGG TGC AGC ATC were used to amplify original VH and VL portions, respectively. The resulting phage display G28-5 mini-library was screened using the hCD40ecto recombinant protein. The CD40-positive phage clone 10 was selected from the mini-library, its gene re-cloned into pOPE101, introduced into XL1Blue E. coli cells (Stratagene), produced by IPTG induction and purified from periplasmic extract by IMAC on Ni-NTA. Panel (b) demonstrates an immunoblot of purified scFv G28 clone 10. The purified protein (20 ng/lane) was separated on 12% PAAG-SDS followed by electrotransfer to a PVDF membrane. The membrane was probed with mAb 9E10 (Sigma) against c-Myc epitope incorporated into the scFv. (c) Construction of CAR/G28 fusion protein. The scFv G28-5-10 cDNA fragment was amplified from pSEX81-G28-5-10 using a pair of primers 5′ CCG AGA TCTATA CAG CTT CAG CAG TCA GGA CCT and 5′ AGC GAG CTCCCG TTT TAT TTC CAG CGT GGT followed by digestion with BglII and SacI. The digested fragment was cloned in frame into the pFBsCARslEGF plasmid[11] replacing the EGF which was excised with BamHI and SacI. The new plasmid, pFBsCARslG28, encoding recombinant shCAR fused with scFv G28-5-10 (CAR/G28) and tagged with internal 6His was then used for generation of the recombinant baculovirus genome, according to recommendations for Bac-toBac baculovirus expression system (Life Technologies, Grand Island, NY, USA). All newly engineered cDNA constructs were examined by sequencing using CEQ2000 automatic sequencer and CEQ dye terminator sequencing kit from Beckman (Fullerton, CA, USA). (c) Immunoblot of purified CAR/G28. The purified protein was separated on 12% PAAG-SDS followed by electrotransfer to a PVDF membrane. The membrane has been probed with rabbit anti-CAR antibodies produced in the laboratory. Horseradish peroxidase (HRP)-labeled secondary antibodies and diaminobenzidine staining were used for binding visualization. (d) CAR/G28 fusion protein is bi-specific. CAR/G28 fusion protein binding to both hCD40ecto and Ad5 knob was examined in ELISA. The antigens were adsorbed on plastic (300 ng/well each) and probed with CAR/G28. Binding was detected with rabbit anti-CAR antibodies (1:3000, produced in-house) followed by HRP-labeled anti-rabbit antibodies and orthophenilenediamine staining. (d) CAR/G28 interaction with hCD40ecto and recombinant Ad5 knob.

ogy Unit, Massachusetts General Hospital and Department of Pathology, Harvard Medical School, Boston, MA, USA). Next, the hCD40ecto cDNA was subcloned into the pET21a plasmid and expressed in BL21(DE) *E. coli*. The hCD40ecto recombinant protein of 19 kDa was purified from the cell lysate and its identity confirmed by immunobloting with polyclonal antibody sc-974 and mAb G28-5 against human CD40 (Figure 2a).

The gene segments coding for VH and VL of the G28-5 mAb were PCR-amplified from the corresponding hybridoma cDNA and ligated with a Yol linker by two-step cloning into pSEX81[14] phagemid, thus creating a miniphage display library that facilitated anti-CD40 scFv isolation. The library was screened using hCD40ecto as described.[15] Positive clones were identified in ELISA, and primary DNA structure of the scFv gene was confirmed by sequencing. Single chain Fv G28-5 cDNA was recloned into expression vector pOPE101[16] used to produce scFv G28-5 of approximately 29 kDa in *E. coli*. Purification of scFv was performed with Ni-NTA as described[17] (Figure 2b). The ability of scFv G28-5 to specifically bind hCD40ecto was demonstrated in ELISA (data not shown).

To generate the CAR/G28 fusion protein, scFv G28-5 cDNA was cloned in-frame with that of CAR, generating the pFBsCARsIG28 plasmid, which was then used for baculovirus genome generation. Our attempts to express CAR/G28 cDNA in various bacterial expression systems failed due to massive protein degradation (data not shown). The baculovirus genome containing the CAR/G28 cDNA was used to transfect Sf-9 insect cells and the resultant baculovirus was used for larger scale infection of High Five cells. The recombinant fusion protein was produced and purified from 1 liter of supernatant of infected cell culture. Purified recombinant fusion protein had the expected molecular weight of 54 kDa as demonstrated by immunoblot with anti-CAR antibodies (Figure 2c). The level of the fusion protein production was approximately 2 mg/l of culture. Specificity of the CAR/G28 towards both recombinant hCD40ecto and Ad5 fiber[18] knob was demonstrated in ELISA (Figure 2d). After confirming the dual specificity of CAR/G28, we examined the recombinant protein for its ability to target Ad infection to DCs of rhesus monkey or human origin. Adenoviruses bearing luciferase (Luc) or green fluorescent protein (GFP) as transgenes were used in the DC transduction experiments.

Rhesus monocyte-derived DCs (RhMDDC) were derived from heparinized peripheral mononuclear cells isolated by gradient centrifugation. Monocytes were enriched to a purity of >80% by adherence to anti-CD14 coated magnetic microbeads and cultured in RPMI containing autologous plasma and cytokines recombinant human GM-CSF and IL-4 as described.[19] The phenotype of MDDCs was assessed by staining for HLA-DR, CD83, CD80 and CD86 with corresponding anti-human antibodies that cross-react with rhesus macaque (data not shown). The RhMDDCs were infected with Ad at MOI of 100 p.f.u./cell (optimal viral dose determined in Ref. 7) in the presence and absence of CAR/G28. Infection efficiency was assessed by measuring the transgene expression. As shown in Figure 3, both luciferase and GFP activity were significantly higher when the Ad infection was mediated by CAR/G28. The fusion protein enhanced DC transduction in a dose-dependent manner (Figure 3a, b). Of note, doses of CAR/G28 higher than 100 ng per 2.5×10^4 DCs caused further transgene expression enhancement, but some cell toxicity was

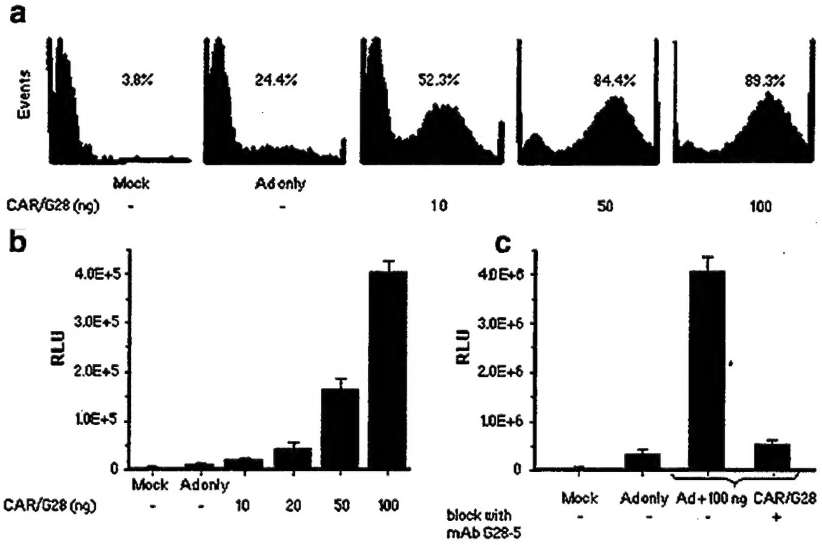

Figure 3 Rhesus DCs transduction with Ad5. Rhesus MDDCs were obtained, briefly, as follows. Mononuclear cells were isolated from normal rhesus heparinized blood by Ficollpaque density gradient centrifugation. Monocytes were enriched by adherence to anti-CD14 magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). Monocyte-enriched cells were cultured at ~1 × 10⁶ cells/ml in RPMI supplemented with 1% autologous plasma, 800 U/ml of granulocyte/macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA, USA) and 1000 U/ml of IL-4 to generate immature MDDCs. On day 6 immature RhMDDCs were infected with Ad5CMVluc (2.5 × 10⁴ cells/experimental point) or Ad5CMVGFP (2 × 10⁵ cells/experimental point) at MOI of 100 or 500, respectively. The virus was pre-incubated with indicated amounts of CAR/G28, before mixing with MDDCs. Transduced MDDCs were analyzed for GFP expression by flow cytometry to measure increased fluorescence intensity (a) or Luc content (b,c) by luminometry to measure relative light units (RLU). Specificity of transduction was demonstrated by inhibition of infection with G28-5 hybridoma supernatant. Mock, RhMDDCs were treated with neither virus, nor fusion protein; Ad only, RhMDDCs were treated with Ad without the fusion protein.

observed at those doses (data not shown). This toxicity effect is probably associated with an unknown effect of soluble CAR. Pre-treatment of DCs with G28-5 hybridoma supernatant efficiently blocked the infection (Figure 3c), indicating that Ad infectivity enhancement was due to engagement of CD40 for virus docking. These results suggest that the dual (human and rhesus) reactivity of CAR/G28 provides an advantage for DC-based vaccine approaches using gene therapy in the nonhuman primate model.

We also evaluated Ad transduction of human DCs with CAR/G28 fusion protein. Human monocyte-derived (HMDDC) were obtained by gradient centrifugation followed by plastic adherence enrichment and cultivation in the presence of cytokines.[20] The HMDDCs were infected with Ad in the presence and absence of indicated amounts of CAR/G28. The ability of CAR/G28 to enhance Ad infectivity towards human DCs was examined with adenovirus vectors bearing either Luc or GFP reporter genes. GFP expression was assessed by direct fluorescence microscopy, whereas the Luc expression was indirectly measured on a luminometer. Data presented in Figure 4 demonstrate CAR/G28-mediated enhancement of Ad transgene expression assayed both qualitatively (Figure 4a–c) and quantitatively (Figure 4e). As a positive control, the Fab-α-CD40 chemical conjugate[7] was used (Figure 4d, f). Analysis of data presented in Figure 4 suggests that the CAR/G28 fusion protein achieves similar levels in promoting DC transduction compared with the Fab-α-CD40 chemical conjugate.

In summary, we describe here a novel recombinant fusion protein designed to promote specific adenovirus-mediated transduction of DCs. After examining prokaryotic and eukaryotic expression systems, we achieved acceptable levels of CAR/G28 recombinant fusion protein production using baculovirus-infected insect cells. The fusion protein has a polyhistidine tag, allowing efficient purification. From the production standpoint, this feature makes the recombinant fusion protein superior in comparison to the anti-Ad:anti-CD40 bispecific antibody Fab-α-CD40 prepared by chemical conjugation. We have demonstrated recently the feasibility of a CAR-ligand fusion protein as an efficient Ad targeting moiety.[11] The present work expands the utility of this kind of CAR-containing molecular adaptor to a new level with scFvs as a fusion partner. Current gene engineering technology allows generation of scFvs against virtually any antigen. The CAR/G28 fusion protein demonstrated the expected dual binding specificity and substantially enhanced Ad gene transfer to DCs (up to 200 times in some experiments compared with untargeted Ad). Monoclonal antibody G28-5 has been shown to be internalized upon CD40 binding.[21] Therefore, we have reason to believe that this internalization process can be involved in CD40-targeted Ad infection of DCs mediated by CAR/G28, in addition to normal, integrin-dependent, viral entry to cells. From a functional perspective, the fusion protein has been tested in two distinct experimental systems using rhesus monkey and human DCs. The cross-reactivity of the CAR/G28 fusion protein with CD40-positive human and monkey DCs resulted in marked enhancement of Ad infection in both systems. These data establish the feasibility of using the nonhuman primate rhesus macaque model for preclinical studies with CAR/G28 to advance gene therapy-based DC vaccine development. In both systems the fusion protein demonstrated outstanding ability to target Ad vectors to DCs efficiently and specifically. The development of

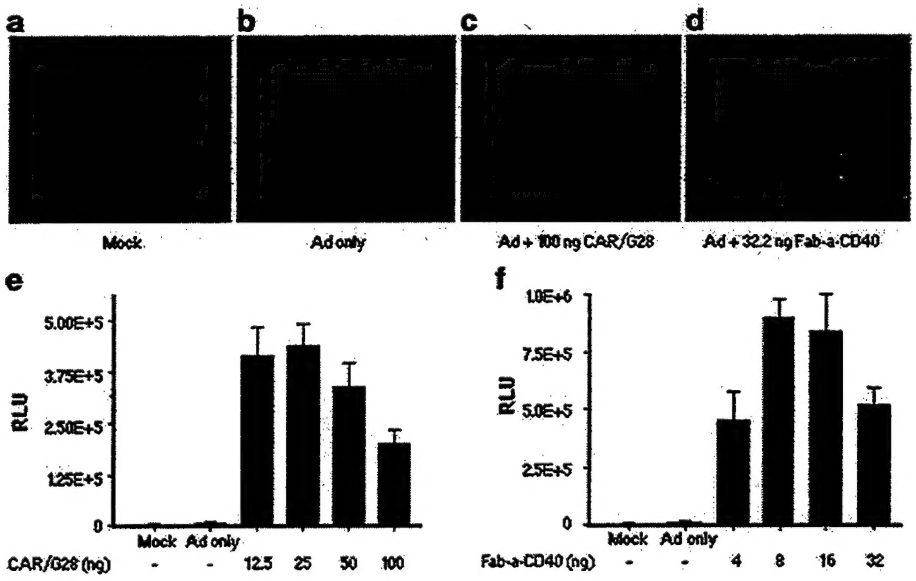

Figure 4 Human DCs transduction with Ad5. Human DCs were generated from peripheral blood monocytes obtained from healthy donors. Monocytes, purified by adherence, were cultured for 6 days in Iscoves's medium (Mediatech Cellgro, Herdon, VA, USA) with 10% human AB serum (Sigma, St Louis, MO, USA) containing 200 ng/ml of interleukin-4 (IL-4; R&D Systems, Minneapolis, MN, USA) and 1000 U/ml of GM-CSF. The resulting DCs were washed in phosphate buffered saline and resuspended at 5 × 10^5/ml in a serum-free medium, macrophage SFM (Gibco BRL, Grand Island, NY, USA) for infection. Human MDDCs were transduced essentially as described in the legend to Figure 3, with the exception that Ad5CMVGFP was used at MOI of 100 and the cells were assayed by fluorescent microscopy. CAR/G28 comparison with Fab-α-CD40 has been done for both viruses. (a) Both CAR/G28 and Fab-α-CD40 potentiate MDDCs transduction with Ad5CMVGFP. (b, c) Both CAR/G28 and Fab-α-CD40 potentiate MDDCs transduction with Ad5CMVluc.

CAR/G28 presents an opportunity to improve existing Ad-based approaches to genetically modify DCs for the treatment of human diseases.

Acknowledgements

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